A METHOD AND KIT FOR THE QUANTITATIVE AND/OR QUALITATIVE DETECTION OF COMPONENTS IN A SAMPLE

FIELD OF THE INVENTION

5   The present invention relates to the field of diagnostic assays and kits for the detection of biological molecules.

BACKGROUND TO THE INVENTION

Micro-arrays are tools for DNA and RNA molecular diagnostics. Detection of nucleic acids is
10   possible using parallelisation techniques thereby enabling the investigation of several thousands of sequences in one reaction or experiment. Most applications focus on expression profiling for measuring adequately the expression of several thousands genes of interest. Detection of molecular binding on micro-arrays is visualized by use of special fluorescent dyes such as Cy3 and Cy5. Visualization of the binding is limited by the stability of
15   the fluorescent marker, and in order to evaluate the processed micro-arrays, highly sophisticated and expensive laser scanning devices are required in addition to highly sophisticated software for analysing the data generated by laser scanning devices.

For the last thirty-five years, metal particles including gold and silver have been used as both contrast enhancement agents or light absorption labels in many different types of analytic
20   and/or diagnostic applications. The great majority of these applications fall under the category of cytoimmunochemistry studies which have used gold or silver enhanced gold particles as markers to study structural aspects of cellular, subcellular, or tissue organization. In these studies, metal particles are usually detected and localized by electron microscopy, including scanning, transmission, and BEI (backscattered electron imaging). These methods take
25   advantage of the electron dense nature of metals or the high atomic number of metals to facilitate the detection of the gold particles by virtue of the large numbers of secondary and backscattered electrons generated by the dense metal (see; Hayat, Immunogold-silver staining reference Page 1 and Chapters 1, 6-15; and Hayat, Colloid Gold reference Chapters 1,5,7 and others).

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A number of patents describe the use of enzymatic methods or gold-based technology on micro-arrays to detect the presence of specific sequences of DNA. PCT patent application number WO 00/72018 (EP 1 179 180) (Advanced Array Technologies) describes the use of

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biotinylated DNA as probes against DNA samples immobilised on a glass micro-array, using gold (10 nm) labelled streptavidin as a visualisation agent.

5   US 2001/0010906A1 describes optimisation of capture probes design for sandwich hybridisation on solid carrier.

EP 1 164 201 describes the use of inverted detection for identifying and/or quantifying nucleotide target sequences on biochips using micro-fluidity techniques.

10   EP 1 096 024 describes a method for detection of homologue sequences after multiplex PCR for detecting Staphylococcus microorganisms.

AU8366001, AU7547501, CA2397280, WO0196604, AU736340, US6214560, CN1282378T, EP1023456, EP1021554, AU1294399, WO9920789 disclose a similar technique using gold
15   labelled streptavidin particles of at least 80 nm for visualisation of bound nucleic acids on a glass micro-array using back scattered light.

US5583001, US196306 and US5731158 disclose the use of *in situ* amplification techniques wherein the signal generated by the bound probe is amplified and visualised using enzymatic
20   or gold based techniques. The catalysed amplification reporter deposit (CARD) technique was found to result in a signal amplification ranging from 10 to 100 fold and found to give equal results as the polymer based technique. The principle of CARD is widely used for several applications including electron microscopy, immunohistochemistry, ELISA, and *in situ* hybridization. The use of gold in a CARD based amplification has been described for the
25   above mentioned techniques but real signal amplification on micro-arrays is not disclosed using gold or an enzymatic based technology.

US 6451980 discloses a technique for signal enhancement of bi-specific antibody-polymer probe for use in immunoassay. Therein is described the use of bispecific antibodies where
30   one part of the antibody complex recognises the antigen and the other part binds the polymer probe consisting of a poly-L-lysine backbone coated with "detectable signals".

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WO0206511 and AU8292001 (Genisphere) disclose an amplification technique applicable on micro-arrays, consisting of a dendrimer-based approach. Dendrimers are DNA molecules marked with a fluorescent dye. The special feature is that those molecules can form three dimensional structures by a type of hybridisation which results in a supermolecule heavily marked with Cy3 or Cy5 molecules. This leads to an enormous amplification of the signal generated by the bound nucleic acid of interest.

EP 1 230 396 and WO 01/36681 (Digene) disclose a technology detecting DNA/RNA hybrids on micro-arrays using a specific monoclonal antibody directed specifically to RNA/DNA hybrids with visualisation using fluorescent dyes.

WO 96/14314 disclose the use of a specific monoclonal antibody detecting DNA/PNA nucleic acid hybrids in solution and on a solid support.

For the detection of low concentrations of molecule components in the field of diagnostics, the methods of chemiluminescence and electrochemiluminescence are widely used. These methods provides a means to detect low concentrations of components by amplifying the number of luminescent molecules or photon generating events manyfold, the resulting "signal amplification" then allowing for detection of low concentration components. However, the above mentioned methods of signal amplification have associated limitations which makes the detection of components by these methods complicated, not easy to use, time consuming, and costly.

Problems of interference of chemical or enzymatic reactions, contamination, complicated and multi-step procedures, limited adaptability to single step "homogeneous" (non-separation) formats, and the requirement of costly and sophisticated instrumentation are areas that those in the art are constantly trying to improve.

Improvements has so far failed to provide means for the quantative and/or qualitative detection of molecules such as DNA, RNA, proteins, polypeptides without an evaluation step requiring an additional device such as a laser scanner, equipment to measure scattered light and/or specialised software. A low cost means of detection of components in samples is of importance in everyday fields of environmental science, veterinary medicine, pharmaceutical

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research, food and water quality control and the like. Furthermore a means which is simple to use, obviates the need for specialised training in equipment and/or protocols. Furthermore, the detection of substances at low concentrations (less than about 1 picomole substance/sample volume analyzed) is presently not possible without the use of fluorescent,

5   luminescent, chemiluminescent, or electrochemiluminescent labels and other detection methods, all of which require optical reading devices to evaluate results.

It is a purpose of this invention not only to overcome the present day limitations, for example the disadvantages of light scattering-based diagnostic assays, but to also overcome the

10   limitations and disadvantages of other non-light scattering methods such as signal amplification. This invention as described herein is easier to use, has greater detection sensitivity, and is capable of measuring components in samples across a wider concentration range than was previously possible. The present invention is broadly applicable to most sample types and assay formats as a signal generation and detection system for

15   components.

The present invention provides a signal and detection system for the detection of components where the procedures can be simplified and the amount and types of steps and reagents reduced. The present invention provides for the quantitative and/or qualitative detection of

20   single or multiple components in a sample. The present invention also provides for substantial reductions in the number of different tests and amounts of sample material that are analysed. Such reduction in the number of individual tests leads to reduced cost and waste production, especially medically-related waste that must be disposed of.

25   **SUMMARY OF THE INVENTION**

One embodiment of the present invention is a method for quantitatively and/or qualitatively detecting one or more components in one or more samples, said component capable of binding to a probe, comprising the steps in the following order:

a) applying one or more samples onto a solid support,

30   b) optionally storing solid supports of step a) at a temperature between 0 and 10 degrees Celsius,

c) incubating solid support of step a) or b) with one or more tagged probes,

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d) incubating solid support with a monoclonal or polyclonal antibody directed against the tag of step c), said antibody raised in species A and said antibody optionally labelled with metal particle,

e) incubating solid support with antibody conjugate, said polymer comprising:

5   - one or more antibodies, anti-A, directed against immunoglobulins of species A, - one or more antibodies, anti-B, directed against immunoglobulins of species B,

- optionally one or more substances which directly or indirectly cause a quantitative colour change compared with the solid support,

f) incubating the solid support with a polypeptide capable recognition by anti-B antibodies,

10   said polypeptide labelled with one or more substances which directly or indirectly cause a quantitative colour change compared with the solid support, and

g) optionally incubating the solid support with a metal enhancement reagent and/or a colour change reagent that is a suitable substrate of an enzyme attached to the antibody conjugate, and

15   h) reading the solid support to quantitatively and/or qualitatively detect said components.

Another embodiment of the present invention is a method as described above wherein step a) is

a) applying one or more probes onto a solid support,

20   and step c) is

c) incubating solid supports with tag-labelled sample,

Another embodiment of the present invention is a method as described above wherein step c) is absent and step d) is

25   d) incubating solid supports with metal-particle-labelled anti-component monoclonal or polyclonal antibody, said antibody raised in species A.

Another embodiment of the present invention is a method as described above further comprising the steps, after step f), of:

30   f-1) repeating steps e) to f), and

f-2) optionally repeating step f-1).

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Another embodiment of the present invention is a method as described above wherein the solid support is supplied with probe pre-applied, and step a) is not performed by the user.

Another embodiment of the present invention is a method as described above wherein the
5   reading of step h) comprises the use of a colour chart.

Another embodiment of the present invention is a method as described above wherein the reading of step h) comprises the use of a device suitable for detecting changes in conductance and/or current across the solid support at the positions at which said samples
10   are applied.

Another embodiment of the present invention is a kit for quantitatively and/or qualitatively detecting one or more components in one or more samples, said component capable of binding to a probe, comprising:
15   a) one or more solid supports,
     b) a container in which a quantity antibody conjugate is present, said conjugate comprising:
              - one or more antibodies, anti-A, directed against immunoglobulins of species A, - one
              or more antibodies, anti-B, directed against immunoglobulins of species B,
              -optionally one or more substances which directly or indirectly cause a quantitative
20   colour change compared with the solid support.

Another embodiment of the present invention is a kit as described above further comprising a container in which a quantity of anti-tag polyclonal or monoclonal antibodies is present, said antibodies raised in species A.
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Another embodiment of the present invention is a kit as described above wherein the solid support is pre-loaded with probes capable of binding to said components.

Another embodiment of the present invention is a kit as described above for use in a method
30   as described above.

Another embodiment of the present invention is a kit according as described above for use in detecting, diagnosing and/or monitoring the progress of a Human Papillomavirus (HPV)

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infection and wherein one or more molecular probes is capable of binding to an HPV component.

Another embodiment of the present invention is a kit as described above wherein said
5   component is a coat polypeptide.

Another embodiment of the present invention is a kit as described above wherein said component is a gene selected from the group consisting of HPV 16, HPV18, HPV 31, HPV 33, HPV 35, HPV 52 and HPV 58.
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Another embodiment of the present invention is a kit as described above for use in detecting, diagnosing and/or monitoring the progress of one or more of the disease states in humans as listed in Table 1, by detecting a polypeptide and/or nucleic acid corresponding to the listed component.
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Another embodiment of the present invention is a kit as described above for use in detecting, diagnosing and/or monitoring the progress infections caused by one or more of one or more of HCV, HIV, HBV, HTLV, mycobacteria, *Staphylococcus aureus*.

20   Another embodiment of the present invention is a kit as described above for use in detecting, diagnosing and/or monitoring the progress neurodegenerative diseases by detecting one or more of beta-amyloids, hTAU, phosphoTAU and APOE.

Another embodiment of the present invention is a kit as described above for use in detecting,
25   diagnosing and/or monitoring the progress of malignant diseases, autoimmunity or allergy related diseases by detecting one or more of ANA, Jo-1, Myeloperoxidase, RNP, Scl-70, Sm, SS-A, IgE, IgG-subclasses and circulating antibodies.

Another embodiment of the present invention is a kit as described above for use in
30   environmental testing of water for bacteria.

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Another embodiment of the present invention is a kit as described above for use in environmental testing of food components for genetically modified components, listeria and salmonella.

5   Another embodiment of the present invention is a method for staining components in cell and/or tissue sections suitable for visualisation using microscopy comprising the steps of:
j) incubating said section with one or more tagged probes directed against a component,
k) incubating said section with metal labelled anti-tag monoclonal or polyclonal antibody, said antibody raised in species A,
10   l) incubating said section with antibody/enzyme polymer, said polymer comprising at least:
- one or more antibodies, anti-A, directed against immunoglobulins of species A, - one or more antibodies, anti-B, directed against immunoglobulins of species B,
-optionally one or more substances which directly or indirectly cause a quantitative colour change,
15   m) incubating the section with a polypeptide capable recognition by anti-B antibodies, said polypeptide labelled with one or more substances which directly or indirectly cause a quantitative colour change, and
n) optionally incubating the section with a metal enhancement reagent and/or a colour change reagent that is a suitable substrate of an enzyme attached to the antibody conjugate.
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Another embodiment of the present invention is a method as described above wherein step j) is absent and step k) is
k) incubating section with metal particle labelled anti-component monoclonal or polyclonal antibody, said antibody raised in species A.
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Another embodiment of the present invention is a method as described above further comprising the steps, after step m), of:
m-1) repeating steps l) to m), and
m-2) optionally repeating step m-1).
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Another embodiment of the present invention is a kit for staining components in cell and/or tissue sections suitable for visualisation using microscopy comprising:

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a container in which a quantity of antibody/enzyme polymer antibody, said polymer comprising at least:

> - one or more antibodies, anti-A, directed against immunoglobulins of species A, - one or more antibodies, anti-B, directed against immunoglobulins of species B,
> - optionally one or more substances which directly or indirectly cause a quantitative colour change.

Another embodiment of the present invention is a kit as described above further comprising a container in which a quantity of anti-tag polyclonal or monoclonal antibodies is present, said antibodies raised in species A.

Another embodiment of the present invention is a kit as described above for use in a method as described above.

Another embodiment of the present invention is a method as described above, and a kit according as described above wherein said metal particle is gold.

Another embodiment of the present invention is a method as described above, and a kit as described above wherein said tag is biotin.

Another embodiment of the present invention is a method as described above, and a kit as described above wherein said polypeptide capable recognition by anti-B antibodies is labelled with gold particles and/or alkaline phosphatase.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is related to a method for the quantitative and/or qualitative detection of components in a sample, using a solid support, an antibody conjugate, a tagged probe directed to said components, and an antibody directed against said tag.

A "sample" as used herein means any sample that contains one or more components to be tested, said components capable of binding to a probe enabling identification of said component. Examples of samples or components include, but are not limited to DNA, cDNA,

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mRNA, RNA, nucleic acids, proteins, polypeptides, glycoproteins, receptors, ligands, metabolites, toxins etc. Other examples of samples include, but are not limited to blood, blood components, other bodily fluids, tissues, drinking water, soil, domestic waste, industrial waste, any food stuff – liquid or solid, crops.

5   A "probe" as used herein means any compound capable of specific binding to a component. For example, a nucleic acid oligomer binding to a gene, a ligand binding to a receptor are examples of probe/component interactions according to the invention. According to the present invention, the affinity of binding between a probe and a component is better than 10 uM, 5 uM, 2 uM, 1 uM, 0.1 uM, 0.01 uM or 1 nM. Examples of probes include but are not

10   limited to nucleic acids, PNAs, proteins, peptides, antibodies, ligands, receptors etc.

A "tag" as used here means any type of substance which is capable of being recognised by an antibody. Examples of such tags include, but are not limited to polypeptides, proteins, polysaccharides, amino acids, vitamins (such as biotin), natural or synthetic substances,

15   enzymes (such as AP and HRP), dyes (such as FITC and TR), nucleic acids, , PNA, DNP, digoxygenin, streptavidin, Psoralen. A "tag" as used here also means a metal, metals or organometallic substances. According to one aspect of the invention, the probe is tagged. According to another aspect of the invention, the sample is tagged. The process of tagging is known to the skilled artisan and can be performed on proteins, peptides or nucleic acids. For

20   example, nucleic acid may be biotinylated by performing a polymerase chain reaction on the sample using biotinylated primer(s) specific for the gene of interest. Peptides and proteins may be biotinylated using biotinylation reagents which, for example, biotinylate the C-terminus, the N-terminus and/or reactive side chains of the protein or peptide. The present invention includes any method of the art or future methods for the tagging of sample.

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By "solid support" herein is meant any solid support which is capable of immobilising components and/or samples. Such solid supports are known in the art and include, but are not limited to, nitrocellulose, glass slides, nylon, silane coated slides, nitrocellulose coated slides, plastics. The solid support preferably comprises nitrocellulose.

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Antibodies as used according to the present invention may be whole antibodies or may be part of an antibody, said part comprising at least the complementary determining region and,

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where necessary, a species-specific region that can be recognised by other antibodies (*e.g.* a constant region).

An "antibody conjugate" refers to a complex comprising a bridging compound with an antibody directed against antibodies raised in one species (anti-A, *e.g.* against part of the constant region of mouse antibodies) and/or an antibody directed against antibodies raised in a different species (anti-B, *e.g.* against part of the constant region of rabbit antibodies). Attachment may be by covalent or non-covalent means. Examples of species against which said attached antibodies may be raised include, but are not limited to rabbit, human, goat, mouse, rat, cow, calf, camel, llama, monkey, donkey, guinea pig, chicken and sheep.

According to an aspect of the invention the bridging compound is a soluble polymeric support such as, for example, dextran, polyethylene glycol, agarose, acrylamide, protein, carbohydrate, any bio-polymer, and synthetic polymer, nucleic acid, PNA, latex or any other known or future polymeric subustance. Optionally also attached to the bridging compound are one or more substances that directly or indirectly cause a quantitative colour change compared with the solid support. Said substances may be coloured, or are capable of inducing colour change. For example, the substance might be a dye, a metal particle, a non-metal particle, or an enzyme that catalyses a colour-change reaction. It is within the scope of the invention that a combination of the aforementioned colour change substances are attached to the bridging compound.

A colour change according to the invention is a change in the colour or intensity of colour of the solid support at the location at which the sample is applied (*e.g.* from white to black, from white to red, from white to grey, from white to blue, etc.)

The anti-tag antibody as used in the present invention can be any antibody or part thereof containing a complementary-determining-region that is capable of binding to the tag. The anti-tag antibody may optionally be labelled with one or more substances that are coloured (*e.g.* a dye, a metal particle), or are capable of inducing colour change (*e.g.* an enzyme that catalyses a colour-change reaction.)

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Examples of suitable dyes according to the invention, include but are not limited to FITC, TR, Cy3, Cy5, Rhodamine, RPE, APC, DAPI, RPE-Cy5, PE, Fast Green, Alexins, Tamra, Joe, Rox, 6-FAM, HEX, TET, Dabcyl, TEG.

5   Examples of suitable metal particles according to the invention, include but are not limited to gold, silver, iron, nickel, gadolinium, lead, uranium, caesium, platinum, rhodium, technetium, tellurium, selenium, silicon (silicium), cupper, tin, rhenium, europium, aluminium, germanium, chromium, cadmium, niobium, titanium, magnesium, manganese, molybdenum, antimony, americium, lithium, wolfram, and all metallic substances conducting or semi-conducting.

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Enzymes that catalyse colour change and that are suitable according to the invention may be any used in a colour change assay such as an ELISA or Western blot, and includes, but is not limited to alkaline phosphatase, horse radish peroxidase beta-galactosidase, luciferase, NADH. Reagents involved in visualising the colour change are known to the skilled

15   addressee and include, but are not limited to DAB, TMB, ABTS, AEC, OPD, Fast Red, fuchsine, AP-blue, AP-orange, BCIP, NBT, pNPP, BCI-NBT, CSPD, CDP-STAR, INT-BCIP.

The present invention is also related to the finding that the use of metal particle-labeled strepavidin or antibodies in a method of the invention, said metal particle having a diameter

20   0.6 to 40 nm together with metal enhancement, surprisingly leads to an improvement in quantitative and/or qualitative colour change, or conductance change.

The present invention is related to the finding that the use of metal-particle-labeled probes, said metal particle having a diameter 0.6 to 40 nm and metal enhancement, in combination

25   with the aforementioned conjugate for staining tissues and cells surprisingly leads to enhanced quantitative and/or qualitative colour change, or conductance change.

By metal enhancement is known to the skilled artisan in the field, and refers to an enhancement of signal by means of metal precipitation due to reduction. Means to enhance

30   the signal include the use of metals such as silver and nickel as secondary reagents.
One embodiment of the present invention is a method for quantitatively and/or qualitatively detecting one or more components in one or more samples, said component capable of binding to a probe, comprising the steps in the order of:

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a) Applying one or more samples onto a solid support,

b) Optionally storing solid support at a temperature between 0 and 10 degrees Celcius,

c) Incubating solid support with one or more tagged probes,

d) incubating solid support with a monoclonal or polyclonal antibody directed against the tag
5   of step c), said antibody raised in species A, and said antibody optionally labelled with metal
particle,

e) Incubating spotted solid support with antibody conjugate, said conjugate comprising:

>   - one or more antibodies, anti-A, directed against immunoglobulins of species A,

>   - one or more antibodies, anti-B, directed against immunoglobulins of species B,

10   >   - optionally one or more substances which directly or indirectly cause a quantitative
>   and/or qualitative colour change compared with the solid support,

f) incubating the solid support of step e) with a polypeptide capable of recognition by anti-B
antibodies, said polypeptide labeled with one or more substances which directly or indirectly
cause a quantitative colour change,

15   g) optionally incubating the solid support with a metal enhancement reagent and/or a colour
change reagent that is a suitable substrate of an enzyme attached to the antibody conjugate,
and

h) reading the solid support to quantitatively and/or qualitatively detect said components.

20   By "applying" as used herein in reference to applying one or more samples or probes to a
solid support, is meant deposition of one or more synthetic or biological substances on a solid
support. The deposition may be by a manual method or by using a device, resulting in an
action including, but not limited to spotting, pipetting, printing, jet printing, dropping etc.

25   By "reading" as used herein means determining from the change in the properties of the solid
support at the position where the sample or probe is applied, the concentration of the
components. According to the invention a change in the property of the solid support may be
a colour change and/or a change in electrical conductivity or electrical current at the position
where the sample or probe is applied.

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Reading may mean using normal vision to ascertain a colour change (e.g. from white to red,
from white to black, from white to grey) on the matrix to determine the presence or absence
of a component i.e. a qualitative reading. Reading may also mean using normal vision to

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ascertain a colour change to determine the concentration of a component *i.e.* a qualitative reading. It is within the scope of the invention that the reading may be taken using a colour chart that allows a comparison of the colour of the sample with that of known concentrations of probe or component. It is within the scope of the invention that a colour change disclosed

5   herein may be read with or without the aid of electronic and optical measuring equipment. For example, a colour change of the solid support may be read by means of a reflectance reader as discussed below.

Using a reflectance reader to measure a colour change leads to accurate measurements and allows the determination of the concentration of the probe or component. The concentration

10   of an unknown component can be calculated by interpolation on a standard curve obtained with several concentrations of probe or component.

Reading may also mean using a device to measure a change of electrical conductivity or electrical current at the position on the solid support where the sample or probe is applied, to

15   determine the concentration of the components. The inventors have found that the use of metal-labelled reagents according to the invention (*e.g.* metal-labeled antibody conjugate, metal-labelled polypeptide capable of recognition by anti-B antibodies) can result in a change of electrical conductivity or electrical current of the solid support. The change can be conveniently and accurately read using a device capable of detecting a change in

20   conductivity and/or current across a solid support. Said device may comprise one or more of the following features: one or more electrical contact probes, circuitry to measure conductivity and/or current, an analogue to digital converter. According to one example, one probe of the device contacts an upper surface of the solid support at the position where the sample or probe is applied, and a second probe contacts the same position on the lower surface; the

25   conductivity and/or current across said probes is measured by the device. According to another example, one probe of the device contacts an upper surface of the solid support at the position where the sample or probe is applied, and the whole of the lower surface of the solid support contacts a conducting plate; the conductivity and/or current across said probe and plate is measured by the device. The latter example has the convenience that the

30   measurement of more than one sample requires movement of only the probe contacting the upper surface.

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In one aspect of the invention, the samples are applied to the solid support without the addition of any extra reagents to the sample prior to the application of sample. In another aspect of the invention, the samples are applied to the solid support after a preconditioning procedure which increases the concentration of salt in said samples. The salt may be any

5   dissociating salt in the art, including, but not limited to sodium chloride, potassium chloride. The preconditioning may comprise the addition of a volume of salt solution of a known concentration to a volume of sample. The preconditioning step may comprise the addition of a volume of salt solution of a known concentration to an unknown volume of sample. The concentration of salt in the sample may be adjusted to lie in the range of 100 mM to 500 mM,

10   500 mM to 1 M, 1 M to 1.5 M, 1.5 M to 2 M, 2 M to 2.5 M, 2.5 M to 3 M, 3 M to 3.5 M, 3.5 M to 4 M, 3.5 M to 5 M, 0.5 M to 2.5 M, 0.5 M to 3 M, 0.5 M to 4 M.

In another aspect of the invention, the sample is applied to the solid support at one or more positions with the same sample at the same relative concentration. In another aspect of

15   the invention, the sample is applied to the solid support at one or more positions at the different relative concentrations. In another aspect of the invention, the sample is applied to the solid support at one or more positions at the same and/or different relative concentrations.

In another aspect of the invention, the temperature at which the solid support on

20   which sample has been applied is optionally stored in step b) is between 0 and 10 degrees Celsius, 2 and 10 degrees, 3 and 10 degrees Celsius, 4 and 10 degrees Celsius, 5 and 10 degrees Celsius, 6 and 10 degrees Celsius, 7 and 10 degrees Celsius, 0 and 5 degrees Celsius, 1 and 5 degrees Celsius, 2 and 5 degrees Celsius, 3 and 5 degrees Celsius, 1 degree Celsius, 2 degrees Celsius, 3 degrees Celsius, 4 degrees Celsius, 5 degrees Celsius,

25   6 degrees Celsius, 7 degrees Celsius, 8 degrees Celsius, 9 degrees Celsius, 10 degrees Celsius.

In another aspect of the invention, when the metal particle is gold, the gold may be of any diameter. In another aspect, the gold may have an average diameter of 0.6, 0.8 nm, 1

30   nm, 2 nm, 3 nm, 4 nm, 5 nm, 6 nm, 7 nm, 8 nm, 9 nm, 10 nm, 11 nm, 12 nm, 13 nm, 14 nm, 15 nm, 16 nm, 17 nm, 18 nm, 19 nm, 20 nm, 21 nm, 22 nm, 23 nm, 24 nm, 25 nm, 26 nm, 27 nm, 28 nm, 29 nm, 30 nm, 31 nm, 32 nm, 33 nm, 34 nm, 35 nm, 36 nm, 37 nm, 38 nm, 39

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nm, 40 nm and 0.6 to 1.0 nm, 1.0 to 5.0 nm, 5.0 to 10 nm, 10 to 15 nm, 15 to 20 nm, 20 to 25 nm, 25 to 30 nm, 30 to 35 nm, 35 to 40 nm.

In another aspect of the invention, after applying the sample in step a), step b) is performed without drying or baking the samples. In another aspect of the invention, the samples applied to the solid support in step a) are allowed to dry. Methods of drying samples are known in the art and can include, but are not limited to, drying in the air, drying in a incubator, drying in an chamber under low pressure optionally heated. According to another aspect of the invention, the samples applied to the solid support are baked by exposed to a temperature of between 60 to 70 degrees Celsius, 65 to 75 degrees Celsius, 70 to 80 degrees Celsius, 75 to 85 degrees Celsius, 80 to 90 degrees Celsius, 65 degrees Celsius, 70 degrees Celsius, 75 degrees Celsius, 80 degrees Celsius, 85 degrees Celsius or 90 degrees Celsius. The exposure time may be for no more than 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes or 60 minutes. The samples may be dried and then baked, only dried, only baked.

The introduction of wash steps in the method above, may be determined by the skilled artisan in accordance with commonly understood protocols in immunoassays such as ELISA and Western blots. For example, one or more wash steps may be introduced after one or more incubation step, using with a washing reagent such as a buffer. Examples of wash steps are provided in the Examples section.

The use of an antibody conjugate as described herein leads to a surprising signal enhancement accompanied by an insignificant or absent increase of background signal which could not have been anticipated by the skilled artisan. Indeed, the inventors have tested other sandwich type assays which do not use the antibody conjugate, and found an accompanying increase in background signal and the introduction of artefacts. The use of the antibody conjugate as disclosed herein, as found by the inventors, provides signal amplification without a concomitant increase in background signal.

The "metal enhancement reagent" of step g) is any metal-containing reagent wherein the metal precipitates due to reduction. Examples include but are not limited to a silver enhancement reagent by Aurion (the Netherlands), BBI (UK), Sigma-Aldrich (USA), or Amersham (UK).

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The "polypeptide capable of recognition by anti-B antibodies" in step f) can be any polypeptide or substance which is recognised by the anti-B antibody attached to the antibody conjugate of step e). If it is a polypeptide, it might be an antibody raised in species B, or an antigenic part thereof. If it is another substance, it might be polysaccharides, amino acid, natural or synthetic substances, nucleic acids, PNA, which is capable of binding to the complimentary determining region of the anti-B antibody of step e).

The components in the samples may be measured qualitatively or quantitatively determined by reading the solid support by measuring a change in property of the solid support at the position where the sample was applied (*e.g.* measuring a colour change and/or a change in electrical conductivity/current). The change in property may be caused by the dye, the metal, the metal after metal enhancement and/or the colour produced after the enzyme catalyses a colour change reagent. It is an aspect of the invention that the change in property is caused by the metal without metal enhancement. It is an aspect of the invention that the change in property is caused by the dye alone. It is an aspect of the invention that the change in property is caused by metal using metal enhancement. It is an aspect of the invention that the change in property is caused by an enzyme-linked colour change. It is an aspect of the invention that the change in property is caused by one or more of the aforementioned aspects.

In another aspect of the invention, the use of tagged-probe as described in a method above is circumvented by using an antibody raised in species A directly against a component in the sample. Thus, in this embodiment of the invention, step c) as described above is absent, and step d) reads:

d) incubating solid supports with metal-particle labelled anti-component monoclonal or polyclonal antibody, said antibody raised in species A.

The use of an antibody directed against a component in a sample obviates the need for an additional step in which a tagged probe is added, and for an additional assay reagent, the introduction of which can lead to handling error.

18

In another aspect of the invention, one or more probes are immobilized onto the solid support and one or more samples applied thereto. Thus, the method is performed as described above including the variations disclosed, with steps substituted or performed identically, as indicated in the method comprising the steps in the following order:

5   a) Applying one or more probes onto a solid support,

b) Optionally storing solid supports of step a) at 0 to 10 degrees Celsius,

c) Incubating solid support with tagged-sample,

d) Incubating solid support with metal-particle-labeled anti-tag monoclonal or polyclonal antibody, said antibody raised in species A,

10   e) Incubating solid support with antibody conjugate, said conjugate comprising:

- one or more antibodies, anti-A, directed against immunoglobulins of species A, - one or more antibodies, anti-B, directed against immunoglobulins of species B,

-optionally one or more substances which directly or indirectly cause a quantitative and/or qualitative colour change compared with the solid support,

15   f) Incubating the solid support with a polypeptide capable recognition by anti-B antibodies, said polypeptide labelled with one or more substances which directly or indirectly cause a quantitative colour change compared with the solid support, and

g) optionally incubating the solid support with a metal enhancement reagent and/or a colour change reagent that is a suitable substrate of an enzyme attached to the antibody conjugate,

20   and

h) reading the solid support to quantitatively and/or qualitatively detect said components.

According to the above embodiment, the sample is tagged. The process of tagging is known to the skilled artisan and can be performed on proteins, peptides or nucleic acids as

25   discussed above.

In another embodiment, the sample is not tagged, and the component bound to the probe is directly detected using a metal-labelled antibody directed to said component. Thus, the need to tag sample is obviated. In one embodiment of the invention, step c) as described

30   above is absent, and step d) reads:

d) incubating solid supports with metal labelled anti-component monoclonal or polyclonal antibody, said antibody raised in species A.

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In another embodiment of the present invention, the solid supports are provided with the probe pre-applied. In one aspect of the invention, the solid support is provided with probe located one or more positions, said probe recognising the same component. In one aspect of the invention, the solid support is provided with probe located one or more positions, said
5   probe recognising different components. Thus, the method of the invention wherein the probe is immobilised onto the solid support is performed from step c). A method in which the solid support is provided with probe pre-applied enables a sample to be assayed for components without the necessity for performing probe application steps. Furthermore, a method using a solid support provided with probe pre-applied, and probe recognising more than one
10   component, enables single samples to be analysed for several components with a single incubation. For example, a single solid support may be used to detect for several cancerous or pre-cancerous conditions as described below by screening a single sample.

It is one advantage of the invention that it does not necessarily require an optical reading device such as a laser scanner or back-scatter measuring equipment, and hence is
15   convenient for use in environments away from laboratory conditions. The invention allows quantitative and/or qualitative results to be obtained at the location at which the sample was taken, for example, in a general practitioner's surgery, in an individual's home, in hospitals, generally 'in the field' without any specialist analytical instruments. Furthermore, the invention provides an assay that is as sensitive as, or more sensitive than assays which use
20   fluorescence. Furthermore, since specialised measuring equipment is not necessarily required, the assay could be performed by a non-specialist.

Another embodiment of the present invention is a method for detecting a component in one or more samples, said method as describe above, further comprising, after step f) the
25   steps of:

f-1) repeating steps e) to f), and

f-2) optionally repeating step f-1).

The steps described in the embodiment above are an amplification, which further
30   enhances the sensitivity of the above method, while minimizing the increase in background signal.

20

Another aspect of the present invention is a kit for the quantitative and/or qualitative detection of components in a sample comprising the following components:

k) one or more solid supports, and

l) a container in which a quantity of antibody conjugate as described above is present.

5

A container may be any sealed or resealable vessel suitable for carrying a quantity of antibody conjugate. Examples include, but are not limited to screw cap vials, push cap vials, break-seal-to-open vials, syringes.

10   A kit according to the present invention allows a skilled artisan to perform one or more steps of the method disclosed herein, in a convenient manner. The kit may allow a method of the present invention to be performed without the need to measure or determine the concentrations of reagents, so enabling a fast and reproducible assaying of one or more samples.

15

Another aspect of the present invention is a kit as described above, comprising a solid support according to item k), wherein said support is pre-spotted with one or more molecular probes. In one aspect of the invention, the solid support is provided with probe located one or more positions, said probe recognising the same component. In one aspect of the invention,

20   the solid support is provided with probe located one or more positions, said probe recognising different components. Thus, a kit supplied with solid support in which the molecular probe is pre-applied enables a sample to be assayed for components without the necessity for performing application steps. Furthermore, a kit supplied with solid support pre-spotted with more than one molecule probe, each capable of recognising a different component enables a

25   single sample to be analysed for several components with a single incubation. For example, a single solid support may be used to detect for several pre-cancerous or cancerous conditions as described below by screening a single sample.

Another aspect of the present invention is a kit as disclosed herein, further comprising

30   one or more metal-labelled probes. Each probe may be specific for a component in a sample to be detected.

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Another aspect of the present invention is a kit as disclosed herein, comprising antibody conjugate.

Another aspect of the present invention is a kit as disclosed herein, anti-tag antibody raised in species A, said antibody optionally labelled with metal particle.

Another aspect of the present invention is a kit as disclosed herein, a polypeptide capable recognition by anti-B antibodies, said polypeptide labelled with one or more substances which directly or indirectly cause a quantitative colour change compared with the solid support.

Another aspect of the present invention is a kit as disclosed herein comprising one or more additional containers in which reagent necessary for visualisation using the colour change enzyme linked to the antibody conjugate are present.

Another aspect of the present invention is a kit as disclosed herein comprising one or more additional containers in which reagent necessary for the tagging of sample to be tested are present. As already mentioned above, method and reagent for tagging of protein and nucleic acids are known in the art.

Another aspect of the invention is a kit as disclosed herein comprising one or more additional containers in which metal-particle-labelled anti-component antibodies are present.

Another aspect of the invention is a kit as disclosed herein comprising one or more additional containers in which a tagged probe(s) capable of binding to a component(s) is present.

Another aspect of the invention is a kit as disclosed herein comprising one or more additional containers in which a polypeptide capable recognition by anti-B antibodies, said polypeptide labelled with one or more substances which directly or indirectly cause a quantitative colour change compared with the solid support is present.

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In another aspect of the invention, the kit enables the skilled person to perform one or more of the method disclosed herein. The kit may comprise one or more additional containers in which reagents are present enabling the skilled person to perform the complete method. Alternatively, the kit may comprise a minimum number of containers, such as only item I), for

5   example, that enables a skilled person to perform the method disclosed herein.

In another aspect of the invention, the kit contains instructions for use. In another aspect of the invention the instructions describing a method of the invention as disclosed herein.

10

In another aspect of the invention, the kit may be used for the diagnosis of disease, susceptibility of disease, monitoring the progress of disease, monitoring the progress of disease during treatment, testing of food, water, soil, testing for contamination, testing for the presence of genetically modified (GM) food components and/or organisms.

15

Another aspect of the present invention is a method and/or kit as disclosed herein for detecting the presence of a component in a sample by visualisation, wherein the sample to be tested comprises a component related to a disease, and the probe is an antibody directed against the DNA, mRNA, cDNA or polypeptide representing said component or part thereof in

20   the diseased individual. Alternatively, the molecular probe is a nucleic acid (DNA, PNA) oligomer which is capable of hybridizing to the DNA, mRNA, cDNA representing said component or part thereof in the diseased individual. A method and/or kit of the invention uses one or more of the embodiments disclosed herein. Examples of components which are associated with diseases and which are detectable using the method and/or kit of the

25   invention are provided in Table 1.

A method and/or kit according to the present invention may be used for the diagnosis and detection of cancer in individuals, for example, for the diagnosis of a type of cancer, for the early detection of cancer, to monitor the progress of cancer in individuals already

30   diagnosed with the disease, to detect a relapse of cancer. Cancer is still a major disease and to prolong life expectancy, it would be advantageous to detect the disease in a pre-clinical stage. A diagnostic assay as disclosed herein makes this possible. Non-limiting examples of components to which cancer or several hereditary conditions associated with are provided in

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Table 1 and, one or more of which are detectable using the method and/or kit of the invention. A diagnosis may require detection of one of more of the listed molecules.

Number	Component	Comments
1.	BRCA1	breast cancer 1, early onset
2.	TP53	tumor protein p53 (Li-Fraumeni syndrome)
3.	CFTR	cystic fibrosis transmembrane conductance regulator, ATP-binding cassette (sub-family C, member 7)
4.	APP	amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease)
5.	APOE	apolipoprotein E
6.	BRCA2	breast cancer 2, early onset
7.	HBB	hemoglobin, beta
8.	APC	adenomatosis polyposis coli
9.	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
10.	HD	huntington (Huntington disease)
11.	BCL2	B-cell CLL/lymphoma 2
12.	ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1
13.	BAX	BCL2-associated X protein
14.	DMD	dystrophin (muscular dystrophy, Duchenne and Becker types), includes DXS142, DXS164, DXS206, DXS230, DXS239, DXS268, DXS269, DXS270, DXS272
15.	CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
16.	ATM	ataxia telangiectasia mutated (includes complementation groups A, C and D)
17.	TNF	tumor necrosis factor (TNF superfamily, member 2)
18.	RB1	retinoblastoma 1 (including osteosarcoma)
19.	VEGF	vascular endothelial growth factor
20.	ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
21.	FGG	fibrinogen, gamma polypeptide
22.	HPRT1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)
23.	MAPT	microtubule-associated protein tau
24.	MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)
25.	RUNX1	runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)
26.	SOD1	superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))
27.	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
28.	PAX6	paired box gene 6 (aniridia, keratitis)
29.	NF1	neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease)
30.	FN1	fibronectin 1
31.	CASP3	caspase 3, apoptosis-related cysteine protease

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Number	Component	Comments
32.	PAH	phenylalanine hydroxylase
33.	GAPD	glyceraldehyde-3-phosphate dehydrogenase
34.	PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
35.	HFE	hemochromatosis
36.	FGFR3	fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)
37.	EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
38.	DSCR1	Down syndrome critical region gene 1
39.	MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
40.	PABPC1	poly(A) binding protein, cytoplasmic 1
41.	CYP3A5	cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 5
42.	PSEN1	presenilin 1 (Alzheimer disease 3)
43.	FBN1	fibrillin 1 (Marfan syndrome)
44.	MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
45.	AKT1	v-akt murine thymoma viral oncogene homolog 1
46.	CCND1	cyclin D1 (PRAD1: parathyroid adenomatosis 1)
47.	MTHFR	5,10-methylenetetrahydrofolate reductase (NADPH)
48.	AR	androgen receptor (dihydrotestosterone receptor; testicular feminization; spinal and bulbar muscular atrophy; Kennedy disease)
49.	TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)
50.	IL6	interleukin 6 (interferon, beta 2)
51.	KRAS2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog
52.	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
53.	RET	ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)
54.	PPARG	peroxisome proliferative activated receptor, gamma
55.	ACTB	actin, beta
56.	CDH1	cadherin 1, type 1, E-cadherin (epithelial)
57.	ESR1	estrogen receptor 1
58.	IGF1	insulin-like growth factor 1 (somatomedin C)
59.	GSTP1	glutathione S-transferase pi
60.	IL8	interleukin 8
61.	LPL	lipoprotein lipase
62.	FMR1	fragile X mental retardation 1
63.	WT1	Wilms tumor 1
64.	IL1B	interleukin 1, beta
65.	CYP1A1	cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1
66.	CTNNB1	catenin (cadherin-associated protein), beta 1 (88kD)
67.	ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)

25

Number	Component	Comments
68.	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
69.	KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
70.	ATP7B	ATPase, Cu++ transporting, beta polypeptide (Wilson disease)
71.	IGF2	insulin-like growth factor 2 (somatomedin A)
72.	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)
73.	CYP2C19	cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase), polypeptide 19
74.	BCR	breakpoint cluster region
75.	FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)
76.	CASP8	caspase 8, apoptosis-related cysteine protease
77.	INSR	insulin receptor
78.	G6PD	glucose-6-phosphate dehydrogenase
79.	IL4	interleukin 4
80.	DRD2	dopamine receptor D2
81.	FGFR1	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)
82.	COL1A1	collagen, type I, alpha 1
83.	BLM	Bloom syndrome
84.	NF2	neurofibromin 2 (bilateral acoustic neuroma)
85.	MMP1	matrix metalloproteinase 1 (interstitial collagenase)
86.	IL2	interleukin 2
87.	GRB2	growth factor receptor-bound protein 2
88.	BCL2L1	BCL2-like 1
89.	PSEN2	presenilin 2 (Alzheimer disease 4)
90.	TNFRSF6	tumor necrosis factor receptor superfamily, member 6
91.	CD44	CD44 antigen (homing function and Indian blood group system)
92.	MMP9	matrix metalloproteinase 9 (gelatinase B, 92kD gelatinase, 92kD type IV collagenase)
93.	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
94.	GSTM1	glutathione S-transferase M1
95.	IL1A	interleukin 1, alpha
96.	MET	met proto-oncogene (hepatocyte growth factor receptor)
97.	ABO	ABO blood group (transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase B, alpha 1-3-galactosyltransferase)
98.	NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
99.	NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)
100.	EGR1	early growth response 1
101.	TTR	transthyretin (prealbumin, amyloidosis type I)
102.	SOD2	superoxide dismutase 2, mitochondrial

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Number	Component	Comments
103.	SCYA2	small inducible cytokine A2 (monocyte chemotactic protein 1)
104.	NOS3	nitric oxide synthase 3 (endothelial cell)
105.	CDC2	cell division cycle 2, G1 to S and G2 to M
106.	STAT1	signal transducer and activator of transcription 1, 91kD
107.	SNCA	synuclein, alpha (non A4 component of amyloid precursor)
108.	CLU	clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)
109.	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
110.	TYR	tyrosinase (oculocutaneous albinism IA)
111.	MADH4	MAD, mothers against decapentaplegic homolog 4 (Drosophila)
112.	CDK2	cyclin-dependent kinase 2
113.	MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatinase)
114.	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
115.	CASP1	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)
116.	PCNA	proliferating cell nuclear antigen
117.	HLA-A, -B, -C	major histocompatibility complex, class I, A , B, C
118.	APOB	apolipoprotein B (including Ag(x) antigen)
119.	CASP9	caspase 9, apoptosis-related cysteine protease
120.	NOS2A	nitric oxide synthase 2A (inducible, hepatocytes)
121.	IFNG	interferon, gamma
122.	APOA1	apolipoprotein A-I
123.	AGT	angiotensinogen (serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 8)
124.	ADA	adenosine deaminase
125.	ICAM1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
126.	CYP19	cytochrome P450, subfamily XIX (aromatization of androgens)
127.	SLC6A4	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4
128.	TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A
129.	CD4	CD4 antigen (p55)
130.	VWF	von Willebrand factor
131.	ACTA1	actin, alpha 1, skeletal muscle
132.	MECP2	methyl CpG binding protein 2 (Rett syndrome)
133.	COMT	catechol-O-methyltransferase
134.	TERT	telomerase reverse transcriptase
135.	PKD	polycystic kidney disease 1 (autosomal dominant)
136.	F7	coagulation factor VII (serum prothrombin conversion accelerator)
137.	PMP22	peripheral myelin protein 22

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Number	Component	Comments
138.	F5	coagulation factor V (proaccelerin, labile factor)
139.	PPARA	peroxisome proliferative activated receptor, alpha
140.	GCK	glucokinase (hexokinase 4, maturity onset diabetes of the young 2)
141.	MUC1	mucin 1, transmembrane
142.	SPP1	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
143.	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
144.	IGF1R	insulin-like growth factor 1 receptor
145.	IL4R	interleukin 4 receptor
146.	DCC	deleted in colorectal carcinoma
147.	PML	promyelocytic leukemia
148.	PDGFRB	platelet-derived growth factor receptor, beta polypeptide
149.	AGTR1	angiotensin receptor 1
150.	UBE3A	ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome)
151.	CREBBP	CREB binding protein (Rubinstein-Taybi syndrome)
152.	CYP1B1	cytochrome P450, subfamily I (dioxin-inducible), polypeptide 1 (glaucoma 3, primary infantile)
153.	AKT2	v-akt murine thymoma viral oncogene homolog 2
154.	PLAT	plasminogen activator, tissue
155.	CHRNA7	cholinergic receptor, nicotinic, alpha polypeptide 7
156.	TIMP1	tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)
157.	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
158.	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)
159.	CDC42	cell division cycle 42 (GTP binding protein, 25kD)
160.	VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor
161.	NTRK1	neurotrophic tyrosine kinase, receptor, type 1
162.	VIM	vimentin
163.	TGFBR2	transforming growth factor, beta receptor II (70-80kD)
164.	DHFR	dihydrofolate reductase
165.	PTCH	patched homolog (Drosophila)
166.	CYP2A6	cytochrome P450, subfamily IIA (phenobarbital-inducible), polypeptide 6
167.	HSPCA	heat shock 90kD protein 1, alpha
168.	E2F1	E2F transcription factor 1
169.	CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit
170.	LCK	lymphocyte-specific protein tyrosine kinase
171.	LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)
172.	RARA	retinoic acid receptor, alpha
173.	PDZK1	PDZ domain containing 1
174.	ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)

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Number	Component	Comments
175.	PAX3	paired box gene 3 (Waardenburg syndrome 1)
176.	FGF2	fibroblast growth factor 2 (basic)
177.	GJB1	gap junction protein, beta 1, 32kD (connexin 32, Charcot-Marie-Tooth neuropathy, X-linked)
178.	LMNA	lamin A/C
179.	CAPN3	calpain 3, (p94)
180.	ADPRT	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase)
181.	TUBB	tubulin, beta polypeptide
182.	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1
183.	IL1RN	interleukin 1 receptor antagonist
184.	CTGF	connective tissue growth factor
185.	GSTT1	glutathione S-transferase theta 1
186.	DRD4	dopamine receptor D4
187.	HTR2A	5-hydroxytryptamine (serotonin) receptor 2A
188.	FHIT	fragile histidine triad gene
189.	ETV6	ets variant gene 6 (TEL oncogene)
190.	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
191.	PPP3R1	protein phosphatase 3 (formerly 2B), regulatory subunit B (19kD), alpha isoform (calcineurin B, type I)
192.	TIMP3	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)
193.	COL1A2	collagen, type I, alpha 2
194.	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
195.	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
196.	ESR2	estrogen receptor 2 (ER beta)
197.	B2M	beta-2-microglobulin
198.	SDF1	stromal cell-derived factor 1
199.	F9	coagulation factor IX (plasma thromboplastic component, Christmas disease, hemophilia B)
200.	MAPK14	mitogen-activated protein kinase 14
201.	BAK1	BCL2-antagonist/killer 1
202.	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
203.	ACTG1	actin, gamma 1
204.	KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
205.	SCTR	secretin receptor
206.	LEPR	leptin receptor
207.	SP1	Sp1 transcription factor
208.	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)
209.	MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
210.	IiL12B	interleukin 12B (natural killer cell stimulatory factor 2,

Number	Component	Comments
		cytotoxic lymphocyte maturation factor 2, p40)
211.	IGF2R	insulin-like growth factor 2 receptor
212.	FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
213.	CD36	CD36 antigen (collagen type I receptor, thrombospondin receptor)
214.	FRD	Friedreich ataxia
215.	COL2A1	collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)
216.	GSN	gelsolin (amyloidosis, Finnish type)
217.	CYP2E	cytochrome P450, subfamily IIE (ethanol-inducible)
218.	APAF1	apoptotic protease activating factor
219.	ANK1	ankyrin 1, erythrocytic
220.	SLC6A3	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3
221.	CASP7	caspase 7, apoptosis-related cysteine protease
222.	MYH7	myosin, heavy polypeptide 7, cardiac muscle, beta
223.	JUNB	jun B proto-oncogene
224.	GHR	growth hormone receptor
225.	IRS1	insulin receptor substrate 1
226.	CASP10	caspase 10, apoptosis-related cysteine protease
227.	BDNF	brain-derived neurotrophic factor
228.	ATP7A	ATPase, Cu++ transporting, alpha polypeptide (Menkes syndrome)
229.	TCF1	transcription factor 1, hepatic; LF-B1, hepatic nuclear factor (HNF1), albumin proximal factor
230.	HGF	hepatocyte growth factor (hepapoietin A; scatter factor)
231.	CYP17	cytochrome P450, subfamily XVII (steroid 17-alpha-hydroxylase), adrenal hyperplasia
232.	PTPN1	protein tyrosine phosphatase, non-receptor type 1
233.	ADRB3	adrenergic, beta-3-, receptor
234.	TNFSF6	tumor necrosis factor (ligand) superfamily, member 6
235.	ERCC5	excision repair cross-complementing rodent repair deficiency, complementation group 5 (xeroderma pigmentosum, complementation group G (Cockayne syndrome))
236.	VCAM1	vascular cell adhesion molecule 1
237.	TF	transferrin
238.	ACE	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
239.	LRP1	low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)
240.	CDK5	cyclin-dependent kinase 5
241.	ACACA	acetyl-Coenzyme A carboxylase alpha
242.	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B
243.	NOTCH3	Notch homolog 3 (Drosophila)
244.	ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3

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Number	Component	Comments
		(avian)
245.	CSK	c-src tyrosine kinase
246.	SCN5A	sodium channel, voltage-gated, type V, alpha polypeptide (long (electrocardiographic) QT syndrome 3)
247.	BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)
248.	FYN	FYN oncogene related to SRC, FGR, YES
249.	CTSK	cathepsin K (pycnodysostosis)
250.	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
251.	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
252.	SCYA5	small inducible cytokine A5 (RANTES)
253.	BMP4	bone morphogenetic protein 4
254.	ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
255.	NR3C1	nuclear receptor subfamily 3, group C, member 1
256.	THBS1	thrombospondin 1
257.	CETP	cholesteryl ester transfer protein, plasma
258.	PTPRC	protein tyrosine phosphatase, receptor type, C
259.	NME1	non-metastatic cells 1, protein (NM23A) expressed in
260.	TGFBI	transforming growth factor, beta-induced, 68kD
261.	SREBF1	sterol regulatory element binding transcription factor 1
262.	MMP14	matrix metalloproteinase 14 (membrane-inserted)
263.	KCNQ1	potassium voltage-gated channel, KQT-like subfamily, member 1
264.	TUBA1	tubulin, alpha 1 (testis specific)
265.	SELE	selectin E (endothelial adhesion molecule 1)
266.	ATRX	alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae)
267.	IL2RG	interleukin 2 receptor, gamma (severe combined immunodeficiency)
268.	IGFBP3	insulin-like growth factor binding protein 3
269.	JAK3	Janus kinase 3 (a protein tyrosine kinase, leukocyte)
270.	CSF1R	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
271.	SHC1	SHC (Src homology 2 domain containing) transforming protein 1
272.	CASP4	caspase 4, apoptosis-related cysteine protease
273.	PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)
274.	CXCR4	chemokine (C-X-C motif), receptor 4 (fusin)
275.	CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
276.	ARHA	ras homolog gene family, member A
277.	SHH	sonic hedgehog homolog (Drosophila)
278.	RARB	retinoic acid receptor, beta
279.	MME	membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)
280.	CA2	carbonic anhydrase II
281.	PRKDC	protein kinase, DNA-activated, catalytic polypeptide

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Number	Component	Comments
282.	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
283.	PRKCA	protein kinase C, alpha
284.	CASP2	caspase 2, apoptosis-related cysteine protease (neural precursor cell expressed, developmentally down-regulated 2)
285.	DMBT1	deleted in malignant brain tumors 1
286.	TGFB2	transforming growth fator, beta 2
287.	TSC2	tuberous sclerosis 2
288.	PSAP	prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)
289.	XPC	xeroderma pigmentosum, complementation group C
290.	THRA	thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian)
291.	ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2 (xeroderma pigmentosum D)
292.	MAPK1	mitogen-activated protein kinase 1
293.	ATP6B1	ATPase, H+ transporting, lysosomal (vacuolar proton pump), beta polypeptide, 56/58kD, isoform 1 (Renal tubular acidosis with deafness)
294.	BAG1	BCL2-associated athanogene
295.	ACHE	acetylcholinesterase (YT blood group)
296.	EGF	epidermal growth factor (beta-urogastrone)
297.	DUSP1	dual specificity phosphatase 1
298.	CASP6	caspase 6, apoptosis-related cysteine protease
299.	THRB	thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)
300.	BAD	BCL2-antagonist of cell death
301.	STAT6	signal transducer and activator of transcription 6, interleukin-4 induced
302.	ELN	elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)
303.	MAOA	monoamine oxidase A
304.	F8	coagulation factor VIII, procoagulant component (hemophilia A)
305.	ENG	endoglin (Osler-Rendu-Weber syndrome 1)
306.	HSPB1	heat shock 27kD protein 1
307.	HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
308.	PIM1	pim-1 oncogene
309.	PON1	paraoxonase 1
310.	AHR	aryl hydrocarbon receptor
311.	ITGB2	integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)
312.	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H

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Number	Component	Comments
		synthase and cyclooxygenase)
313.	PLCG1	phospholipase C, gamma 1 (formerly subtype 148)
314.	APOC3	apolipoprotein C-III
315.	NRG1	neuregulin 1
316.	CD14	CD14 antigen
317.	IRF1	interferon regulatory factor 1
318.	ALPL	alkaline phosphatase, liver/bone/kidney
319.	ALDOA	aldolase A, fructose-bisphosphate
320.	XPA	xeroderma pigmentosum, complementation group A
321.	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide
322.	IL5	interleukin 5 (colony-stimulating factor, eosinophil)
323.	BMP2	bone morphogenetic protein 2
324.	GSK3A	glycogen synthase kinase 3 alpha
325.	STK11	serine/threonine kinase 11 (Peutz-Jeghers syndrome)
326.	GSK3B	glycogen synthase kinase 3 beta
327.	CRYBB1	crystallin, beta B1
328.	STAT5A	signal transducer and activator of transcription 5A
329.	SCA1	spinocerebellar ataxia 1 (olivopontocerebellar ataxia 1, autosomal dominant, ataxin 1)
330.	RXRA	retinoid X receptor, alpha
331.	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
332.	MMP13	matrix metalloproteinase 13 (collagenase 3)
333.	TSHR	thyroid stimulating hormone receptor
334.	MT2A	metallothionein 2A
335.	TSSC3	tumor suppressing subtransferable candidate 3
336.	RHO	rhodopsin (opsin 2, rod pigment) (retinitis pigmentosa 4, autosomal dominant)
337.	GADD45A	growth arrest and DNA-damage-inducible, alpha
338.	LCAT	lecithin-cholesterol acyltransferase
339.	GSR	glutathione reductase
340.	TOP2A	topoisomerase (DNA) II alpha (170kD)
341.	GPX1	glutathione peroxidase 1
342.	FLT3	fms-related tyrosine kinase 3
343.	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
344.	TPM1	tropomyosin 1 (alpha)
345.	ABCA4	ATP-binding cassette, sub-family A (ABC1), member 4
346.	KCNH2	potassium voltage-gated channel, subfamily H (eag-related), member 2
347.	HNF4A	hepatocyte nuclear factor 4, alpha
348.	DPYD	dihydropyrimidine dehydrogenase
349.	MADH2	MAD, mothers against decapentaplegic homolog 2 (Drosophila)
350.	AFP	alpha-fetoprotein
351.	TIMP2	tissue inhibitor of metalloproteinase 2
352.	ITK	IL2-inducible T-cell kinase

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Number	Component	Comments
353.	ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)
354.	SCYA4	small inducible cytokine A4
355.	GCGR	glucagon receptor
356.	TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)
357.	MYB	v-myb myeloblastosis viral oncogene homolog (avian)
358.	LTA	lymphotoxin alpha (TNF superfamily, member 1)
359.	LIF	leukemia inhibitory factor (cholinergic differentiation factor)
360.	CYBB	cytochrome b-245, beta polypeptide (chronic granulomatous disease)
361.	CTSL	cathepsin L
362.	BCL2A1	BCL2-related protein A1
363.	TFRC	transferrin receptor (p90, CD71)
364.	RALGDS	ral guanine nucleotide dissociation stimulator
365.	CYP2C8	cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase), polypeptide 8
366.	CD38	CD38 antigen (p45)
367.	PRKCZ	protein kinase C, zeta
368.	LAMR1	laminin receptor 1 (67kD, ribosomal protein SA)
369.	IL12A	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)
370.	FGA	fibrinogen, A alpha polypeptide
371.	EEF1A1	eukaryotic translation elongation factor 1 alpha 1
372.	CYP21A2	cytochrome P450, subfamily XXIA (steroid 21-hydroxylase, congenital adrenal hyperplasia), polypeptide 2
373.	CSF2	colony stimulating factor 2 (granulocyte-macrophage)
374.	TNFRSF5	tumor necrosis factor receptor superfamily, member 5
375.	MBP	myelin basic protein
376.	PTK2	PTK2 protein tyrosine kinase 2
377.	KLK3	kallikrein 3, (prostate specific antigen)
378.	GALT	galactose-1-phosphate uridylyltransferase
379.	APEX	APEX nuclease (multifunctional DNA repair enzyme)
380.	EPHB2	EphB2
381.	BIK	BCL2-interacting killer (apoptosis-inducing)
382.	SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1
383.	IL2RA	interleukin 2 receptor, alpha
384.	IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)
385.	AXL	AXL receptor tyrosine kinase
386.	ADRB1	adrenergic, beta-1-, receptor
387.	RAD51	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)
388.	GJA1	gap junction protein, alpha 1, 43kD (connexin 43)
389.	EWSR1	Ewing sarcoma breakpoint region 1
390.	CCR2	chemokine (C-C motif) receptor 2

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Number	Component	Comments
391.	RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)
392.	CTNNA1	catenin (cadherin-associated protein), alpha 1 (102kD)
393.	MYO7A	myosin VIIA (Usher syndrome 1B (autosomal recessive, severe))
394.	F3	coagulation factor III (thromboplastin, tissue factor)
395.	EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)
396.	CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)
397.	ENO1	enolase 1, (alpha)
398.	TGFBR1	transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kD)
399.	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
400.	ANPEP	alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal aminopeptidase, CD13, p150)

TABLE 1: List of components which are disease-related and are detectable using the kit and/or method of the present invention.

A kit and/or method according to the invention may be used to detect infectious diseases. Some infectious diseases are life threatening and can appear in combination with other infections. Thus, the sooner they can be detected and characterised, the sooner an appropriate therapy can be established and better for the patient. A kit and/or method as disclosed above can be used to detect said infectious agents. Components which may be detected according to the kit and/or method are those which form part of the infectious agent and/or are produced by the infectious agent. Viruses in diseased individuals detectable according to the kit and/or method include, but are not limited to HCV, HIV, HBV, HTLV, HPV (see also oncology). Bacteria in diseased individuals detectable according to the kit and/or method include, but are not limited to mycobacteria, syphilis, *Staphylococcus aureus* (screening of MRSA).

A kit and/or method according to the invention may be used to detect neurodegenerative diseases. Components which may be detected are those involved in degenerative diseases and include, but are not limited to beta-amyloids (Alzheimer's disease), hTAU, phosphoTAU and APOE.

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A kit and/or method according to the invention may be used to detect prion-related diseases. Diseases associated with prions include Kreutzfeld Jacob disease and BSE.

A kit and/or method according to the invention may be used to detect diseases related to autoimmunity. Components which may be detected are those involved in autoimmunity include, but are not limited to ANA, Jo-1, Myeloperoxidase, RNP, Scl-70, Sm, SS-A.

A kit and/or method according to the invention may be used to detect diseases related to allergy. Components which may be detected are those involved in allergy and include, but are not limited to IgE, IgG-subclasses and circulating antibodies.

A kit and/or method according to the invention may be used in the field of genomics to detect susceptibility to disease, possibility of passing conditions to offspring, single nucleotide polymorphisms etc. Examples of fields in which a kit and/or method of the invention apply include, but are not limited to HLA typing, p53 polymorphism (SNP) related to the sensitivity of developing a cervix carcinoma after an HPV 16 infection, hypertension, detection of polymorphism in relation to the susceptibility for osteoporosis, detection of mutations in Factor V (Leiden), detection of the genetic susceptibility for SIDS (cot death), hereditary: paternity tests, etc., detection of micro satellite instability, detection of the success rate of therapy related to cessation of smoking, detection of disturbances in the metabolism of lipids including cholesterol (HDL, LDL, VLDL and their receptors) in relationship to cardiovascular diseases such as atheromathosis, detection of genomic defects related to obesitas, detection of genomic defects related to diabetes, detection of mutations associated with drug resistance (to HIV, etc..), screening and detection of systic fibrosis (CFTR mutations), detection of mutations in the mitochondrial genome related to a number of diseases as: neurogenic muscular weakness, retinitis pigmentosa, ocular myopathy, etc.

A kit and/or method according to the invention may be used in the fields related to environmental testing. Many applications are related to water where it is important to have a technology which is sensitive enough to detect very small amounts of contaminants or unwanted compounds in reasonably economical manner. Examples of environmental tests include:

- checking (monitoring) of yeast infections in swimming pool water

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- monitoring of biological pollution in general
- biological contaminants in potable water (amoebae, coliform bacteria, etc..).

In addition to water testing, the environmental testing for genetically modified
organisms may be performed according to a kit and/or method of the present invention.
Genetically Modified Organisms can be detected, or samples screened for the absence of.
Checking for possible modifications is sometimes difficult, however, a sensitive technique
such as that provided by the present method is suitable for such a purpose.

A kit and/or method according to the invention may be used to detect the infection of
food. Inspection of all places and objects related to food needs sensitive methods, and kit
and/or method of the present invention provide the required sensitivity. Furthermore, a kit
and/or method of the present invention can be performed and the results obtained at the site
at which the inspection takes place, so obviating the need to send samples to a lab. Thus,
steps can be taken immediately if necessary. Examples of the agents that may be detected
include Listeria, Salmonella, prions (for BSE). Molecules which may be detected assay are
those which form part of the agent and/or are produced by the agent.

A kit and/or method of the present invention may be applied in standard biochemical
detection protocols. All the existing types of blotting techniques show an enhancement in
sensitivity using the method disclosed herein, without the requirement for radioisotopes or
chemilluminescent detection such as photographic plates, or phosphor screens. It is also
possible to use the method in combination with image analysis. Examples of blotting
protocols that may use methods of the present invention include but are not limited to
Western blotting, Northern blotting, Southern blotting, vacuum blotting, contact blotting,
reversed line blot and related techniques, dot blotting, micro-arrays, macro-arrays.

Another embodiment of the present invention is a method for staining sections of cells
and/or tissues suitable for visualisation using microscopy. Types of microscopy may be any,
and include, but are not limited to light microscopy, tunneling electron microscopy, scanning
electron microscopy, transmission electron microscopy.

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According to one aspect of the invention, a method for staining components in cell and/or tissue sections, said staining suitable for visualisation using microscopy comprises the following steps:

n) incubating section with one or more tagged probes directed against a component,

5   m) incubating section with metal-particle-labeled anti-tag monoclonal or polyclonal antibody, said antibody raised in species A,

o) incubating section with an antibody conjugate, said conjugate comprising:

      - one or more antibodies, anti-A, directed against immunoglobulins of species A,

      - one or more antibodies, anti-B, directed against immunoglobulins of species B,

10   - optionally one or more substances which directly or indirectly cause a quantitative colour change,

p) incubating the section with a polypeptide capable recognition by anti-B antibodies, said polypeptide labelled with one or more substances which directly or indirectly cause a quantitative colour change, and

15   q) optionally incubating the section in a metal enhancement reagent and/or a colour change reagent that is a suitable substrate of an enzyme attached to the antibody conjugate.

The antibody conjugate is already defined herein. The introduction of other steps into the method, such as wash steps, for example, may be known by the skilled artisan practicing

20   in the field of immunohistochemistry. The "metal enhancement reagent" of step q) is already defined herein for step i). The sections described above are visualised by observing the dye, by observing the metal, by metal enhancement and/or by making use of the enzyme that catalyses the colour change attached to the antibody conjugate. It is an aspect of the invention to visualise the samples using metal enhancement alone. It is an aspect of the

25   invention to visualise the samples by observing the dye alone. It is an aspect of the invention to visualise the samples by observing the metal without metal enhancement. It is an aspect of the invention to visualise the samples using only enzyme-linked colour change. It is an aspect of the invention to visualise the samples using one or more of the aforementioned visualisations.

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In another aspect of the invention, the use of tagged probe as described in a method above is circumvented by using an antibody raised in species A directly against a component

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in the section. Thus, in this embodiment of the invention, step n) as described above is absent, and step m) reads:

m) incubating section with metal-particle labeled anti-component monoclonal or polyclonal antibodies, said antibodies raised in species A.

5

Another embodiment of the present invention is a method for detecting a component in a section, said method as describe above, further comprising, after step p) the steps of:

p-1) repeating steps o) to p), and

p-2) optionally repeating step p-1).

10

The steps described in the embodiment above are an amplification, which further enhances the sensitivity of the above method, while minimizing the increase in background signal.

15

Another embodiment of the present invention is a kit for staining sections of cells and/or tissues suitable for visualisation using microscopy comprising the following components:

q) a container in which a quantity of antibody conjugate as described above is present.

20

A container may be any sealed or resealable vessel as described elsewhere herein.

A kit for staining sections according to the present invention allows a skilled artisan to perform one or more steps of the method disclosed herein, in a convenient manner. The kit may allow a method of the present invention to be performed without the need to measure or

25

determine the concentrations of reagents, so enabling a fast and reproducible staining of sections.

Another aspect of the present invention is a kit for staining sections as disclosed herein, further comprising one or more metal-particle-labelled probes. Each probe may be

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specific for a component in a section to be detected.

Another aspect of the present invention is a kit as disclosed herein, comprising antibody conjugate.

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Another aspect of the present invention is a kit as disclosed herein, anti-tag antibody raised in species A, said antibody optionally labeled with metal particle.

5   Another aspect of the present invention is a kit as disclosed herein comprising one or more additional containers in which reagent necessary for visualisation using the colour change enzyme linked to the antibody conjugate are present.

Another aspect of the present invention is a kit as disclosed herein comprising one or 10   more additional containers in which reagent necessary for performing metal enhancement are present.

Another aspect of the present invention is a kit as disclosed herein, a polypeptide capable recognition by anti-B antibodies, said polypeptide labelled with one or more 15   substances which directly or indirectly cause a quantitative colour change.

Another aspect of the invention is a kit as disclosed herein comprising one or more additional containers in which metal-particle-labelled anti-component antibodies are present.

20   Another aspect of the invention is a kit as disclosed herein comprising one or more additional containers in which a tagged probe(s) capable of binding to a component(s) is present.

Another aspect of the invention is a kit as disclosed herein comprising one or more 25   additional containers in which a polypeptide capable recognition by anti-B antibodies, said polypeptide labelled with one or more substances which directly or indirectly cause a quantitative colour change is present

In another aspect of the invention, the kit for staining sections enables the skilled 30   person to perform one or more of the method disclosed herein. The kit may comprise one or more additional containers in which reagents are present enabling the skilled person to perform the complete method. Alternatively, the kit may comprise a minimum number of

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containers, such as only item q), for example, that enables a skilled person to perform the method disclosed herein.

5   In another aspect of the invention, the kit contains instructions for use. In another aspect of the invention the instructions describe a method of the invention as disclosed herein.

A method and/or kit of the present invention for staining sections of cells may equally well be performed on any cell or tissue in the applications of flow cytometry and *in situ*
10   hybridisation, wherein the visualisation of cells and tissues is necessary. Due to the sensitivity of the method as disclosed herein, target antigens (proteins and other substances) can be visualised in tissues and cells with antibodies and, using *in situ* hybridization, they can be visualised by use of nucleic acid probes.

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EXAMPLES

Section 1: Materials and methods

Oligonucleotides

5   Target: 5' GGATTATTGTTAAATATTGATAAGGAT 3'

Visualisation oligo: 5' ATCCTTATCAATATT 3'

Oligo op drager: 5' TAACAATAATCC 3'

The above mentioned oligonucleotides are derived from the Anthrax lethal factor genome.

10   **Nylon or nitrocellulose coated slides**

Coated slides such as Nytran coated slides, nitrocellulose coated slides were purchased from Schleicher and Schuell and printed with DNA capturing oligonucleotides using a Microcast micro-arrayer.

Mofified DNA oligonucleotides were custom made by Qbiogene (France) and in varying
15   concentrations dissolved in printing buffer (6x SSC).

Micro-arrays were manufactured by printing varying oligonucleotide concentrations ranging from 0.001 µM to 20 µM on the coated glass slide. Negative controls consisted of printing buffer without DNA oligonucleotides and printing buffer with a DNA oligonucleotide complementary to a non-related sequence in the same concentration as the capturing
20   oligonucleotide of interest. After drying during 30 minutes at room temperature, the slides were baked at 80 °C during 30 minutes. DNA printed at Nytran coated slides was cross linked using UV radiation according to previously described protocols.

Slides were stored dustfree at 4°C until further analysis.

25   **Silane coated slides**

Coated slides such as Nytran coated slides, nitrocellulose coated slides were purchased from Schleicher and Schuell and printed with DNA oligonucleotides using a Microcast micro-arrayer.

DNA oligonucleotides were custom made by Qbiogene (France) and in varying
30   concentrations dissolved in printing buffer. Silane coated slides and modified oligonucleotides were activated according to previously described protocols.

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Micro-arrays were manufactured by printing varying oligonucleotide concentrations ranging from 0.001 μM to 20 μM on the activated silane coated glass slide. After drying during 30 minutes at room temperature, the printed slides were stored dustfree at 4°C.

Classical glass slides

5   Classical microscopical glass slides were washed with bidistilled water followed by immersion in a 10 % NaOH solution at room temperature after an ultrasound treatment during 30 minutes. After several washes in running tap water, slides were washed several times with bidistilled water. Afterwards the slides were dried at 80 °C.

Modified oligonucleotides were acivated and coupled with aminosilane according to
10   previously described protocols (Kumar et al. Silanized nucleic acids: a general platform for DNA immobilization. Nucleic acids res 2000; 28: e71.).

The silanized oligonucleotides were dissolved in printing buffer (50% DMSO,) and printed as described above.

Printing of the micro-arrays

15   Micro-arrays were printed with each concentration in sixfold, capturing oligonucleotides as well as the above described appropriate negative controls.

Section 2: Experiments to compare methods of detection using gold and enzyme visualisations in combination with polymer amplification and other technologies

20   **Part 1 - Visualization experiments of labeled oligonucleotides attached at the above described solid assays**

Experiment 1 – part 1: visualization of labeled oligonucleotides using streptavidin

Micro-arrays were printed as described previously. All concentrations ranging from 0.001 μM to 20 μM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and
25   stored dust-free at 4°C until use.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA (SERVA, Germany).

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were incubated with streptavidin/alkaline phosphatase(concentration 1/1000 in
30   PBS/BSA buffer) (Roche Germany) during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

The alkaline phosphatase reaction was developed by incubating the slides with napthol substrate in appropriate buffer (Dako, Denmark) during 30 minutes at room temperature.

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Experiment 2 – part 1: visualization of labeled oligonucleotides using streptavin labeled with gold particles

Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and

5   stored dust-free at 4°C until use.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution for 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA

10   for 5 minutes.

Slides were incubated with streptavidin/gold 0.8 nm (concentration 1/50 in washing buffer) or 6 nm (concentration 1/20 in washing buffer) (Aurion, the Netherlands) for 120 minutes followed by six washes with washing buffer at room temperature.

Slides were rinsed triplefold for five minutes with PBS, followed by distilled water.

15   The gold particles were visualized by silver enhancement (Aurion, the Netherlands) for 15 minutes.

Experiment 3 – part 1: visualization of labeled oligonucleotides using monoclonal antibodies labeled with gold particles

20   Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution for 30 minutes at room

25   temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) for 5 minutes.

Slides were incubated with monoclonal antibody/gold 0.8 nm (concentration 1/50 in washing buffer), 6 nm (concentration 1/20 in washing buffer) or 40 nm during 120 minutes followed by

30   six washes with washing buffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

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Experiment 4 – part 1: visualization of labeled oligonucleotides using polyclonal antibodies labeled with gold particles

Micro-arrays were printed like described previously. All concentrations ranging from 0.001 μM to 20 μM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and

5   stored dust-free at 4°C until use.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA)

10   during 5 minutes.

Slides were incubated with monoclonal antibody/gold 0.8 nm (concentration 1/50 in washing buffer) or 6 nm (concentration 1/20 in washing buffer) during 120 minutes followed by six washes with washing buffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

15   The gold particles were visualized by silver enhancement during 15 minutes.

Experiment 5 – part 1: visualization of labeled oligonucleotides using monoclonal and polyclonal antibodies in a polymer enhanced amplification technique visualized enzymatically

Micro-arrays were printed like described previously. All concentrations ranging from 0.001

20   μM to 20 μM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

25   Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes and incubated with the same washing buffer .

Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer at room temperature.

30   Slides were incubated with dextran polymer coated with numerous anti-mouse antibodies and anti-rabbit antibodies and labeled with alkaline phosphatase enzym for 30 minutes followed by six washes with washing buffer.

45

The alkaline phosphatase reaction was developed by incubating the slides with napthol substrate in appropriate buffer during 30 minutes at room temperature.

Experiment 6 – part 1: visualization of labeled oligonucleotides using gold labeled monoclonal
5   **and polyclonal antibodies in a polymer enhanced amplification technique**
Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.
Slides were washed twice with PBS (pH 7.4) supplemented with BSA.
10   Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.
Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes and incubated with the same washing buffer .
Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles
15   ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer at room temperature.
Slides were incubated with dextran polymer coated with numerous anti-mouse antibodies and anti-rabbit antibodies for 30 minutes followed by six washes with washing buffer.
Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles
20   ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer at room temperature
Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.
The gold particles were visualized by silver enhancement during 15 minutes.

25   **Experiment 7 – part 1: visualization of labeled oligonucleotides using CARD amplification**
technology visualized enzymatically
Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.
30   Slides were washed twice with PBS (pH 7.4) supplemented with BSA.
Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

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Slides were incubated with streptavidin/peroxidases (concentration 1/500 in PBS/BSA buffer) during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

The slide was incubated with biotinylated tyramine diluted 1/50 in PBS solution supplemented with 0.03 % H_2O_2 for 10 minutes followed by three washes with wash buffer during 5 minutes.

5   Slides were incubated with streptavidin/alkaline phosphatase (concentration 1/1000 in PBS/BSA buffer) during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

The alkaline phosphatase reaction was developed by incubating the slides with napthol substrate in appropriate buffer during 30 minutes at room temperature.

10

Experiment 8 – part 1: visualization of labeled oligonucleotides using CARD amplification technology visualized with gold labeled streptavidin

Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and

15   stored dust-free at 4°C until use.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were incubated with streptavidin/peroxidases (concentration 1/500 in PBS/BSA buffer)

20   during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

The slide was incubated with biotinylated tyramine diluted 1/50 in PBS solution supplemented with 0.03 % H_2O_2 for 10 minutes followed by three washes with wash buffer during 5 minutes.

Slides were incubated with streptavidin/gold 0.8 nm (concentration 1/50 in washing buffer) or 6 nm (concentration 1/20 in washing buffer) during 120 minutes followed by six washes with

25   washing buffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

47

Experiment 9 – part 1: visualization of labeled oligonucleotides using CARD amplification technology visualized with gold labeled monoclonal or polyclonal antibodies

Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and

5   stored dust-free at 4°C until use.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were incubated with streptavidin/peroxidases (concentration 1/500 in PBS/BSA buffer)

10   during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

The slide was incubated with biotinylated tyramine diluted 1/50 in PBS solution supplemented with 0.03 % H_2O_2 for 10 minutes followed by three washes with wash buffer during 5 minutes.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes.

15   Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles ranging from 0.6 nm to 40 nm during 120 minutes followed by six washes with washingbuffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

20

Part 2: Hybridization experiments of labeled oligonucleotides attached at the above described solid assays

Experiment 2.1 - part 2: visualization of labeled oligonucleotides using streptavidin

Micro-arrays were printed like described previously. All concentrations ranging from 0.001

25   µM to 20 µM were spotted in sixfold including adequate positive and negative controls. Micro-arrays were baked at 80°C during 30 minutes and stored dust free at 4°C until use.

The slides were prehybridized with hybridization mixture supplemented with sonicated herring sperm DNA (150 µg/5 ml hybridization mixture) for 2 hours at room temperature.

Hybridization assay was set up using target DNA and/or visualisation oligonucleotide in a

30   concentration of 250 ng/ml hybridization mixture. Hybridization was carried out overnight at 37°C.

Slides were washed twice with 2x SSC at room temperature.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

48

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were incubated with streptavidin/alkaline phosphatase (concentration 1/1000 in PBS/BSA buffer) during 60 minutes followed by three washes with PBS/BSA buffer at room

5   temperature.

The alkaline phosphatase reaction was developed by incubating the slides with napthol substrate in appropriate buffer during 30 minutes at room temperature.

Experiment 2.2 - part 2: visualization of labeled oligonucleotides using streptavin labeled with

10   **gold particles**

Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.

The slides were prehybridized with hybridization mixture supplemented with sonicated herring

15   sperm DNA (150 µg/5 ml hybridization mixture) for 2 hours at room temperature.

Hybridization assay was set up using target DNA and/or visualisation oligonucleotide consisting of labeled oligonucleotide in a concentration of 250 ng/ml hybridization mixture. Hybridization was carried out overnight at 37°C.

Slides were washed twice with 2x SSC at room temperature.

20   Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes.

25   Slides were incubated with streptavidin/gold 0.8 nm (concentration 1/50 in washing buffer) or 6 nm (concentration 1/20 in washing buffer) during 120 minutes followed by six washes with washing buffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

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49

Experiment 3 - part 2: visualization of labeled oligonucleotides using monoclonal antibodies labeled with gold particles

Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and

5   stored dustfree at 4°C until use.

The slides were prehybridized with hybridization mixture supplemented with sonicated herring sperm DNA (150 µg/5 ml hybridization mixture) for 2 hours at room temperature.

Hybridization assay was set up using target DNA and/or visualisation oligonucleotide consisting of labeled oligonucleotide in a concentration of 250 ng/ml hybridization mixture.

10   Hybridization was carried out overnight at 37°C.

Slides were washed twice with 2x SSC at room temperature

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

15   Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes.

Slides were incubated with monoclonal antibody/gold 0.8 nm (concentration 1/50 in washing buffer) or 6 nm (concentration 1/20 in washing buffer), or 40 nm for 120 minutes followed by six washes with washing buffer at room temperature.

20   Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

Experiment 4- part 2: visualization of labeled oligonucleotides using polyclonal antibodies labeled with gold particles

25   Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.

The slides were prehybridized with hybridization mixture supplemented with sonicated herring sperm DNA (150 µg/5 ml hybridization mixture) for 2 hours at room temperature.

30   Hybridization assay was set up using target DNA and/or visualisation oligonucleotide consisting of labeled oligonucleotide in a concentration of 250 ng/ml hybridization mixture. Hybridization was carried out overnight at 37°C.

Slides were washed twice with 2x SSC at room temperature

50

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA)
5   during 5 minutes.

Slides were incubated with monoclonal antibody/gold 0.8 nm (concentration 1/50 in washing buffer) or 6 nm (concentration 1/20 in washing buffer) during 120 minutes followed by six washes with washing buffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.
10   The gold particles were visualized by silver enhancement during 15 minutes.

Experiment 5 - part 2: visualization of labeled oligonucleotides using monoclonal and polyclonal antibodies in a polymer enhanced amplification technique visualized enzymatically

Micro-arrays were printed like described previously. All concentrations ranging from 0.001
15   µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.

The slides were prehybridized with hybridization mixture supplemented with sonicated herring sperm DNA (150 µg/5 ml hybridization mixture) for 2 hours at room temperature.

Hybridization assay was set up using target DNA and/or visualisation oligonucleotide
20   consisting of labeled oligonucleotide in a concentration of 250 ng/ml hybridization mixture. Hybridization was carried out overnight at 37°C.

Slides were washed twice with 2x SSC at room temperature

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room
25   temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes and incubated with the same washing buffer .

Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer
30   at room temperature.

Slides were incubated with dextran polymer coated with numerous anti-mouse antibodies and anti-rabbit antibodies and labeled with alkaline phosphatase enzym for 30 minutes followed by six washes with washing buffer.

51

The alkaline phosphatase reaction was developed by incubating the slides with napthol substrate in appropriate buffer during 30 minutes at room temperature.

Experiment 6 - part 2: visualization of labeled oligonucleotides using gold labeled monoclonal and polyclonal antibodies in a polymer enhanced amplification technique

Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.

The slides were prehybridized with hybridization mixture supplemented with sonicated herring sperm DNA (150 µg/5 ml hybridization mixture) for 2 hours at room temperature.

Hybridization assay was set up using target DNA and/or visualisation oligonucleotide consisting of labeled oligonucleotide in a concentration of 250 ng/ml hybridization mixture. Hybridization was carried out overnight at 37°C.

Slides were washed twice with 2x SSC at room temperature

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes and incubated with the same washing buffer .

Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer at room temperature.

Slides were incubated with dextran polymer coated with numerous anti-mouse antibodies and anti-rabbit antibodies for 30 minutes followed by six washes with washing buffer.

Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer at room temperature

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

52

Experiment 7- part 2: visualization of labeled oligonucleotides using CARD amplification technology visualized enzymatically

Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and

5   stored dust-free at 4°C until use.

The slides were prehybridized with hybridization mixture supplemented with sonicated herring sperm DNA (150 µg/5 ml hybridization mixture) for 2 hours at room temperature.

Hybridization assay was set up using target DNA and/or visualisation oligonucleotide consisting of labeled oligonucleotide in a concentration of 250 ng/ml hybridization mixture.

10   Hybridization was carried out overnight at 37°C.

Slides were washed twice with 2x SSC at room temperature

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

15   Slides were incubated with streptavidin/peroxidases (concentration 1/500 in PBS/BSA buffer) during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

The slide was incubated with biotinylated tyramine diluted 1/50 in PBS solution supplemented with 0.03 % H_2O_2 for 10 minutes followed by three washes with wash buffer during 5 minutes.

Slides were incubated with streptavidin/alkaline phosphatase(concentration 1/1000 in

20   PBS/BSA buffer) during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

The alkaline phosphatase reaction was developed by incubating the slides with napthol substrate in appropriate buffer during 30 minutes at room temperature.

25   **Experiment 8- part 2: visualization of labeled oligonucleotides using CARD amplification technology visualized with gold labeled streptavidin**

Micro-arrays were printed like described previously. All concentrations ranging from 0.001 µM to 20 µM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.

30   The slides were prehybridized with hybridization mixture supplemented with sonicated herring sperm DNA (150 µg/5 ml hybridization mixture) for 2 hours at room temperature.

53

Hybridization assay was set up using target DNA and/or visualisation oligonucleotide consisting of labeled oligonucleotide in a concentration of 250 ng/ml hybridization mixture. Hybridization was carried out overnight at 37°C.

Slides were washed twice with 2x SSC at room temperature

5   Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were incubated with streptavidin/peroxidases (concentration 1/500 in PBS/BSA buffer) during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

10   The slide was incubated with biotinylated tyramine diluted 1/50 in PBS solution supplemented with 0.03 % H_2O_2 for 10 minutes followed by three washes with wash buffer during 5 minutes.

Slides were incubated with streptavidin/gold 0.8 nm (concentration 1/50 in washing buffer) or 6 nm (concentration 1/20 in washing buffer) during 120 minutes followed by six washes with washing buffer at room temperature.

15   Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

Experiment 9- part 2: visualization of labeled oligonucleotides using CARD amplification technology visualized with gold labeled monoclonal or polyclonal antibodies

20   Micro-arrays were printed like described previously. All concentrations ranging from 0.001 μM to 20 μM were spotted in sixfold. Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.

The slides were prehybridized with hybridization mixture supplemented with sonicated herring sperm DNA (150 μg/5 ml hybridization mixture) for 2 hours at room temperature.

25   Hybridization assay was set up using target DNA and/or visualisation oligonucleotide consisting of labeled oligonucleotide in a concentration of 250 ng/ml hybridization mixture. Hybridization was carried out overnight at 37°C.

Slides were washed twice with 2x SSC at room temperature

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

30   Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were incubated with streptavidin/peroxidases (concentration 1/500 in PBS/BSA buffer) during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

54

The slide was incubated with biotinylated tyramine diluted 1/50 in PBS solution supplemented with 0.03 % H_2O_2 for 10 minutes followed by three washes with wash buffer during 5 minutes. Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes.

5   Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles ranging from 0.6 nm to 40 nm during 120 minutes followed by six washes with washing buffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

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<u>Results and discussion</u>

Results of experiments without signal amplification:

Experiment 1 - Visualization with streptavidin-enzyme

Hybridization was visualized as deep red or dark brown spots according to the used enzyme

5   and substrate. The spots were easy discernible at the white background of the slide. Background staining was not present. Visualization was achieved with spotted molecular probe concentration ranging from 0.2 mM to 0.02 mM.

Experiment 2 - Visualization with streptavidin gold 0.8 nm and 6 nm

10   Hybridization was visualized as deep black grey spots according to the used silver enhancement. The spots were easy discernible at the white background of the slide. Background staining was not presented. Visualization was achieved with spotted molecular probe concentration ranging from 0.2 mM to 0.02 mM.

15   **Experiment 3 - Visualization with monoclonal mouse antibodies with gold 0.8 nm and 6 nm**

Hybridization was visualized as deep black spots according to the used silver enhancement. The spots were easy discernible at the white background of the slide. Background staining was not present. Visualization was achieved with spotted molecular probe concentration ranging from 0.2 mM to 0.002 mM. However the signal was stronger with the 6 nm gold

20   compared to the 0.8 nm gold and sharper than with the streptavidin-gold method. In comparison with the polyclonal antibodies the signal was slightly sharper.

Experiment 3 - Visualization with monoclonal mouse antibodies with gold 40 nm

Hybridization was faintly visualized as light rosa spots only at the highest spot concentration

25   of 0.2 mM. Silver enhancement did not yield significant signal strenghtening. Background staining was considerable.

Experiment 4 - Visualization with polyclonal goat antibodies with gold 0.8 nm and 6 nm

Hybridization was visualized as deep black spots according to the used silver enhancement.

30   The spots were easy discernible at the white background of the slide. Background staining was not present. Visualization was achieved with spotted molecular probe concentration ranging from 0.2 mM to 0.002 mM. However the signal was stronger with the 6 nm gold compared to the 0.8 nm gold and sharper than with the streptavidin-gold method.

56

Results of experiments with signal amplification - CARD-amplification with short spacer:

Experiment 7 - Visualization with streptavidin-enzyme

Hybridization was visualized as deep red or dark brown spots according to the used enzym

5   and substrate. The spots were easy discernible at the white background of the slide.
Background staining was moderate to strong. Visualization was achieved with spotted
molecular probe concentration ranging from 0.2- mM to 0.0002 mM.

Experiment 8 - Visualization with streptavidin gold 0.8 nm and 6 nm

10   Hybridization was visualized as deep black grey spots according to the used silver
enhancement. The spots were easy discernible at the white background of the slide.
Background staining was moderate. Visualization was achieved with spotted molecular probe
concentration ranging from 0.2 mM to 0.0002 mM.

15   **Experiment 9 - Visualization with polyclonal goat antibodies with gold 0.8 nm and 6 nm**

Hybridization was visualized as deep black spots according to the used silver enhancement
strategy. The spots were easy discernible at the white background of the slide. Background
staining was moderate. Visualization was achieved with spotted molecular probe
concentration ranging from 0.2 mM to 0.0002 mM. However the signal was stronger with the

20   6 nm gold compared to the 0.8 nm gold and sharper than with the streptavidin-gold method.

Experiment 9 - Visualization with monoclonal mouse antibodies with gold 0.8 nm and 6 nm

Hybridization was visualized as deep black spots according to the used silver enhancement.
The spots were easy discernible at the white background of the slide. Background staining

25   was moderately present. Visualization was achieved with spotted molecular probe
concentration ranging from 0.2 mM to 0.002 mM. However the signal was stronger with the 6
nm gold compared to the 0.8 nm gold and sharper than with the streptavidin-gold method. In
comparison with the polyclonal antibodies the signal was slightly sharper.

30   **Experiment 9 - Visualization with monoclonal mouse antibodies with gold 40 nm**

Hybridization was faintly visualized as light brown spots only at the highest spot concentration
of 0.2 mM. Silver enhancement did not yield significant signal enhancement. Background
staining was considerable.

57

Results of experiments with signal amplification - CARD-amplification with long spacer:

Experiment 7 - Visualization with streptavidin -enzyme

Hybridization was visualized as deep red or dark brown spots according to the used enzyme
and substrate. The spots were easy discernible at the white background of the slide.
Background staining was moderate . Visualization was acieved with spotted molecular probe
concentration ranging from 0.2 mM to 0.0002 mM.

Experiment 8 - Visualization with streptavidin gold 0.8 nm and 6 nm

Hybridization was visualized as deep black grey spots according to the used silver
enhancement. The spots were easy discernible at the white background of the slide.
Background staining was slight. Visualization was achieved with spotted molecular probe
concentration ranging from 0.2 mM to 0.0002 mM.

Experiment 9 - Visualization with polyclonal goat antibodies with gold 0.8 nm and 6 nm

Hybridization was visualized as deep black spots according to the used silver enhancement.
The spots were easy discernible at the white background of the slide. Background staining
was very slight. Visualization was achieved with spotted molecular probe concentration
ranging from 0.2 mM to 0.0002 mM. However the signal was stronger with the 6 nm gold
compared to the 0.8 nm gold and sharper than with the streptavidin-gold method.

Experiment 9 - Visualization with monoclonal mouse antibodies with gold 0.8 nm and 6 nm

Hybridization was visualized as deep black spots according to the used silver enhancement.
The spots were easy discernible at the white background of the slide. Background staining
was very slight. Visualization was achieved with spotted molecular probe concentration
ranging from 0.2 mM to 0.002 mM. However the signal was stronger with the 6 nm gold
compared to the 0.8 nm gold and sharper than with the streptavidin-gold method.   In
comparison with the polyclonal antibodies the signal was slightly sharper.

Experiment 9 - Visualization with monoclonal mouse antibodies with gold 40 nm

Hybridization was faintly visualized as light brown spots only at the highest spot concentration
of 0.2 mM. Silver enhancement did not yield significant signal strenghtening. Background
staining was considerable.

58

Results of experiments with signal amplification - amplification using polymertechnology:

Experiment 5 - Visualization with monoclonal antibodies/polymer labeled with alkaline phosphatase

5   Hybridization was visualized as deep red spots. The spots were easy discernible at the white background of the slide. Background staining was slight. Visualization was achieved with spotted molecular probe concentration ranging from 0.1 mM to 0.0001 mM.

Experiment 6 - Visualization with polyclonal goat antibodies with gold 0.8 nm, 6 nm and 40
10   **nm/polymer/ monoclonal mouse antibodies 0.8 nm, 6 nm and 40 nm**
       Hybridization was visualized as deep black spots according to the used silver enhancement. The spots were easy discernible at the white background of the slide. Background staining was very slight. Visualization was achieved with spotted molecular probe concentration ranging from 0.1 mM to 0.0001 mM. However the signal was stronger with the 0.8 nm gold
15   compared to the 6 nm gold and sharper than with the streptavidin-enzym method. The 40 nm gold particles gave a slight rosa reaction barely discernible with the naked eye.

Experiment 6 - Visualization with monoclonal mouse antibodies 0.8 nm, 6 nm, 40 nm/polymer/polyclonal goat antibodies with gold 0.8 nm, 6 nm and 40 nm
20   Hybridization was visualized as deep black spots according to the used silver enhancement. The spots were easy discernible at the white background of the slide. Background staining was not present. Visualization was achieved with spotted molecular probe concentration ranging from 0.1 mM to 0.0001 mM. However the signal was stronger with the 0.8 nm gold compared to the 6 nm gold and sharper than with the streptavidin-enzyme method. The 40
25   nm gold particles gave a slight rosa reaction barely discernible with the naked eye.

Experiment 6 - Visualization with monoclonal goat antibodies with gold 0.8 nm, 6 nm, 40 nm/polymer/ monoclonal mouse antibodies 0.8 nm, 6 nm and 40 nm
       Hybridization was visualized as deep black spots according to the used silver enhancement.
30   The spots were easy discernible at the white background of the slide. Background staining was very slight. Visualization was achieved with spotted molecular probe concentration ranging from 0.1 mM to 0.0001 mM. However the signal was stronger with the 0.8 nm gold compared to the 6 nm gold and sharper than with the streptavidin-enzym method. The 40 nm gold particles gave a slight rosa reaction barely discernible with the naked eye.

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Experiment 6 - Visualization with monoclonal mouse antibodies with gold 40 nm /polymer/ monoclonal mouse antibodies with gold 40 nm

Hybridization was faintly visualized as light red spots only at the highest spot concentration of 0.2 mM. Silver enhancement did not yield significant signal strengthening. Background
5   staining was considerable.

Section 3: Detection and subtyping of HPV DNA

Micro-arrays were printed like described previously using specific oligonucleotides detecting HPV 16, HPV18, HPV 31, HPV 33, HPV 35, HPV 52 and HPV 58. The oligonucleotides were
10 dissolved in printing buffer resulting in an end concentration of 10 µM and were spotted in sixfold including adequate positive and negative controls using a Microcast micro-arrayer.

This results in a micro-array with 7 seven rows, each row consisting of six identical spots, representing the seven above described HPV types. The negative controls consisted of printing buffer without DNA and a second negative control consisted of an oligonucleotide
15 coding for a non-related gene segment and were printed as two rows consisting of six spots where one row consisted out of printing buffer without DNA and another row consisted of non-related DNA oligonucleotide.

The positive control consisted of an equimolar mixture of the above described HPV type specific oligonucleotides printed in one row consisting of six identical spots.
20 After printing the micro-arrays were air dried at room temperature for 15 minutes.

Micro-arrays were baked at 80°C during 30 minutes and stored dust-free at 4°C until use.

Hybridization with PCR labeled amplification product

Hybridization assay was set up using PCR amplified HPV DNA .
25 During the PCR reaction the amplification product was labeled using a biotin labeled primer.

In another experiment the PCR was set up with two unlabeled primers.

Ten microliter of the PCR amplification product was denatured with 10 µl denaturation solution (NAOH/EDTA). The denatured DNA solution was added to 2 ml of hybridization mixture. The micro-arrays were covered with a cover slip and hybridized overnight at 37°C in
30 a humid chamber.

Slides were washed with SSC buffer.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

60

The hybridization of the biotin labeled PCR product was revealed using one of the methods described above.

Visualisation with streptavidin-alkaline phosphatase

5   Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were incubated with streptavidin/alkaline phosphatase (concentration 1/1000 in PBS/BSA buffer) during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

10 The alkaline phosphatase reaction was developed by incubating the slides with napthol substrate in appropriate buffer during 30 minutes at room temperature.

Visualisation with streptavidin- gold

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

15 Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes.

Slides were incubated with streptavidin/gold 0.8 nm (concentration 1/50 in washing buffer) or

20 6 nm (concentration 1/20 in washing buffer) during 120 minutes followed by six washes with washing buffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

25 **Visualisation with mono-or polyclonal anti-biotin antibody**

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA)

30 during 5 minutes.

Slides were incubated with monoclonal or polyclonal antibody/gold 0.8 nm (concentration 1/50 in washing buffer) or 6 nm (concentration 1/20 in washing buffer) during 120 minutes followed by six washes with washing buffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

61

The gold particles were visualized by silver enhancement during 15 minutes.

Visualisation using signal amplification with polymer technology

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

5   Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes and incubated with the same washing buffer .

Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles

10   ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer at room temperature.

Slides were incubated with dextran polymer coated with numerous anti-mouse antibodies and anti-rabbit antibodies for 30 minutes followed by six washes with washing buffer.

Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles

15   ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer at room temperature

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

20   **Visualisation using signal amplification with CARD technology**

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were incubated with streptavidin/peroxidases (concentration 1/500 in PBS/BSA buffer)

25   during 60 minutes followed by three washes with PBS/BSA buffer at room temperature.

The slide was incubated with biotinylated tyramine diluted 1/50 in PBS solution supplemented with 0.03 % H_2O_2 for 10 minutes followed by three washes with washbuffer during 5 minutes.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes.

30   Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles ranging from 0.6 nm to 40 nm during 120 minutes followed by six washes with washing buffer at room temperature.

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

62

The gold particles were visualized by silver enhancement during 15 minutes

Hybridization with unlabeled PCR amplification product, visualization using anti-DNA antibody, signal amplification with polymer technology and gold.

5   Hybridization assay was set up using PCR amplified HPV DNA .

In this part of the experiment the PCR was set up with two unlabeled primers.

Ten microliter of the PCR amplification product was denatured with 10 µl denaturation solution (NAOH/EDTA). The denatured DNA solution was added to 2 ml of hybridization mixture. The micro-arrays were covered with a cover slip and hybridized overnight at 37°C in

10   a humid chamber.

Slides were washed twice with 2x SSC supplemented with 0.1% SDS at room temperature.

Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

The hybridization of the unlabeled PCR product with its capture oligonucleotide at the micro-array was revealed using an anti-DNA antibody.

15   Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes and incubated with the same washing buffer .

20   Slides were incubated with anti-DNA during 60 minutes followed by six washes with washing buffer at room temperature.

Slides were incubated with dextran polymer coated with anti-mouse antibodies and anti-rabbit antibodies for 30 minutes followed by six washes with washing buffer.

Slides were incubated with monoclonal or polyclonal antibody labeled with gold particles

25   ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer at room temperature

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

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Results and discussion

Hybridized micro-arrays showed areas with very sharp black or red coloured spots in some areas depending on the used substrate. Other areas did not show any signal. Background signal was completely absent.

5   Hybridization was revealed as easy discernible bright spots coloured deep red when using the alkaline phophatase technique for visualization or as deep grey to black spots when using gold-silver enhancement technology. The results were easily evaluated with the naked eye. The best results were obtained with gold labeled antibodies used in a polymer amplification technique.

10   Negative controls did not show any sign of positivity. The positive control was strongly positive. In one experiment the presence of HPV 16 was revealed and in another experiment the presence of HPV 18 was highlighted. Cross-hybridization with other molecular probes was not noted. Samples without HPV DNA did not give a signal at the micro-array.

15   **Section 4: Application of immunohistochemistry: visualization of tissue antigens with of monoclonal antibodies using gold labeled monoclonal and polyclonal antibodies in a polymer enhanced amplification technique**

Sections of 5 μm were cut from paraffin embedded formalin fixed tissue of squamous lung carcinoma and adhered to poly-l-lysine coated glass slides, dried at 55°C overnight and
20   stored dust-free at room temperature. The sections were deparaffinized in two rinses of xylene substitute, followed by rehydratation in an descending series of alcohols down to deionized water. Slides were washed twice with PBS (pH 7.4) and incubated with anti-ema (epithelial membrane antigen) monoclonal mouse antibody (Dakopatts Denmark) according to the instructions of the manufacturer). Slides were washed twice with PBS (pH 7.4)
25   supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes and incubated with the same washing buffer .

30   Slides were incubated with monoclonal or polyclonal antibody labelled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer at room temperature.

64

Slides were incubated with dextran polymer coated with anti-mouse antibodies and anti-rabbit antibodies for 30 minutes followed by six washes with washing buffer.

Slides were incubated with monoclonal or polyclonal antibody labelled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer

5   at room temperature

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes.

The results showed clear and sharp staining with excellent contrast.

10   **Section 5**

5.1 Detection of specific antibodies

Micro-arrays were printed as described previously using specific antigens such as CEA (carcino-embronal antigen) and EMA (epithelial membrane antigen). The antigens were diluted in printing buffer consisting of PBS (pH 7.4) resulting in various concentrations and

15   were spotted in sixfold including adequate positive and negative controls using a Microcast micro-arrayer.

This results in a micro-array with 7 seven rows, each row consisting of six identical spots, representing various concentrations of the above described antibodies or antigens. The negative controls consisted of printing buffer without antigen and a second negative control

20   consisted of a non-related antigen and were printed as two rows consisting of six spots where one row consisted out of printing buffer without antibody or antigen and another row consisted of non-related antigen.

The positive control consisted of an equimolar mixture of antibodiesdirected against the above described antigens printed in one row consisting of six identical spots.

25   After printing the micro-arrays were air dried at room temperature for 15 minutes and stored dust-free at 4°C until use.

Slides were washed twice with PBS (pH 7.4) supplemented with 3% BSA followed by an incubation during 30 minutes at room temperature followed by incubation with anti-CEA (carcino-embryonal antigen) antibody. Slides were washed twice with PBS (pH 7.4)

30   supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

65

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes and incubated with the same washing buffer .

Slides were incubated with monoclonal or polyclonal antibody labelled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washingbuffer

5   at room temperature.

Slides were incubated with dextran polymer coated with numerous anti-mouse antibodies and anti-rabbit antibodies for 30 minutes followed by six washes with washing buffer.

Slides were incubated with monoclonal or polyclonal antibody labelled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer

10   at room temperature

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement during 15 minutes. The results showed clear and sharp staining with excellent contrast.

15   **5.2 Detection of specific antigens**

Micro-arrays were printed like described previously using specific antibodies detecting CEA. The antibodies were diluted in printing buffer consisting of PBS (pH 7.4) resulting in various concentrations and were spotted in sixfold including adequate positive and negative controls using a Microcast micro-arrayer.

20   This results in a micro-array with 7 seven rows, each row consisting of six identical spots, representing various concentrations of the above described antibodies. The negative controls consisted of printing buffer without antibody or antigen and a second negative control consisted of a non-related antigen d were printed as two rows consisting of six spots where one row consisted out of printing buffer without antibody or antigen and another row

25   consisted of non-related antibody or antigen.

The positive control consisted of an equimolar mixture of the above described antigen printed in one row consisting of six identical spots.

After printing the micro-arrays were air dried at room temperature for 15 minutes and stored dust free at 4°C until use.

30   Slides were washed twice with PBS (pH 7.4) and incubated with CEA antigen. Slides were washed twice with PBS (pH 7.4) supplemented with BSA.

Slides were incubated with the same PBS/BSA buffer solution during 30 minutes at room temperature.

66

Slides were washed twice with special washing buffer (PBS pH 7.4 supplemented with BSA) during 5 minutes and incubated with the same washing buffer .

Slides were incubated with monoclonal or polyclonal antibody labelled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer

5   at room temperature.

Slides were incubated with dextran polymer coated with numerous anti-mouse antibodies and anti-rabbit antibodies for 30 minutes followed by six washes with washing buffer.

Slides were incubated with monoclonal or polyclonal antibody labelled with gold particles ranging from 0.6 nm to 40 nm during 60 minutes followed by six washes with washing buffer

10   at room temperature

Slides were rinsed triplefold during five minutes with PBS, followed by distilled water.

The gold particles were visualized by silver enhancement for 15 minutes.

The results showed clear and sharp staining with excellent contrast.